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Role of white adipose lipolysis in the development of NASH induced by methionine-and choline-deficient diet

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Abstract

Methionine- and choline-deficient diet (MCD) is a model for nonalcoholic steatohepatitis (NASH) in rodents. However, the mechanism of NASH development by dietary methionine/choline deficiency remains undetermined. To elucidate the early metabolic changes associated with MCD-NASH, serum metabolomic analysis was performed using mice treated with MCD and control diet for three days and one week, revealing significant increases in oleic and linoleic acids after MCD treatment. These increases were correlated with reduced body weight and white adipose tissue (WAT) mass, increased phosphorylation of hormone-sensitive lipase, and up-regulation of genes encoding carboxylesterase 3 and β2-adrenergic receptor in WAT, indicating accelerated lipolysis in adipocytes. The changes in serum fatty acids and WAT by MCD treatment were reversed by methionine supplementation, and similar alterations were detected in mice fed a methionine-deficient diet (MD), thus demonstrating that dietary methionine deficiency enhances lipolysis in WAT. MD treatment decreased glucose and increased fibroblast growth factor 21 in serum, thus exhibiting a similar metabolic phenotype as the fasting response. Comparison between MCD and choline-deficient diet (CD) treatments suggested that the addition of MD-induced metabolic alterations, such as WAT lipolysis, to CD-induced hepatic steatosis promotes liver injury. Collectively, these results demonstrate an important role for dietary methionine deficiency and WAT lipolysis in the development of MCD-NASH.

Keywords

fasting response; linoleic acid; lipolysis; metabolomics; oleic acid; choline deficiency

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD), defined by the presence of hepatic steatosis regardless of no ethanol consumption, is a common chronic liver disease that is increasing worldwide [1]. NAFLD is classified into two categories, simple steatosis (SS) and nonalcoholic steatohepatitis (NASH), according to the liver histology [2]. The clinical course and outcome differ between SS and NASH, where SS has a benign clinical course, while NASH can develop into liver cirrhosis, hepatic failure, and hepatocellular carcinoma [3]. Several studies to clarify the pathogenesis of NASH using animal models and clinical trials regarding the treatment of NASH have been published [4–7]. The pathogenesis of NASH as a two-hit model was proposed in 1998 and has been widely accepted [8]. The first hit is singular triglyceride (TG) accumulation in hepatocytes resulting in hepatic steatosis and increasing the sensitivity of the liver to the second hits that causes hepatocyte damage, inflammation, and fibrosis. Considering clear differences in the clinical features between steatosis and steatohepatitis, it is of great value to clarify the mechanism of the progression from steatosis to steatohepatitis.

Methionine- and choline-deficient diet (MCD) is a conventional and useful model to induce NASH in rodents. In 1942, Gyorgy and Goldblatt reported that supplementation of choline or methionine reduced the incidence of rats having hepatic steatosis, hepatocyte necrosis, and cirrhosis induced by low casein diet, showing an important role of these nutrients in the pathogenesis of nutritional liver injury [9]. Mice treated with MCD show macrovesicular steatosis, hepatitis, hepatocyte ballooning, and enhancement of pro-inflammatory cytokines and oxidative stress in the liver within a few weeks, and the longer treatment results in hepatic fibrosis [10]. These pathologies are similar to those found in human NASH. Although this model is not accompanied by obesity and insulin resistance, such close similarities prompt the use of MCD for determining the mechanism of NASH. It is recognized that choline-deficient diet (CD) treatment causes hepatic steatosis in mice, mainly due to impaired secretion of very-low-density lipoprotein (VLDL) from the liver [11]. However, the mechanism on how dietary methionine/choline deficiency contributes to the NASH development is not fully understood.

Metabolomics using ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) is a useful method to detect global metabolic alterations in the physiological/pathological conditions in an unbiased manner [12]. Previous studies revealed alterations in phospholipid and bile acid metabolism in mouse models of NASH as a result of enhanced inflammatory signaling [10]. However, the early metabolic changes related to the occurrence of MCD-NASH were not determined. In this study, serum metabolomic analysis of mice treated with MCD for three days and one week was conducted using UPLC-ESI-QTOFMS. Serum metabolomics uncovered significant increases in oleic and linoleic acids after the MCD treatment due to enhanced lipolysis in white adipose tissue (WAT). Increased WAT lipolysis was caused by dietary methionine deficiency. Comparison between MCD and CD treatments suggested that the addition of metabolic changes caused by dietary methionine deficiency, such as enhanced WAT lipolysis, to CD-induced hepatic steatosis promotes liver injury. Induction of lipolysis by a 36-hour fasting to CD-treated mice resulted in the increases in serum

alanine aminotransferase (ALT) levels, an indicator of hepatocyte injury. These findings provide new insight into the role of dietary methionine deficiency and WAT lipolysis in the progression to MCD-NASH.

2. Methods

2.1. Mice and treatment

All studies were conducted according to Institute of Laboratory Animal Resource guidelines and approved by the National Cancer Institute Animal Care and Use Committee. The mice were housed in a specific pathogen-free environment controlled for temperature and light (25 °C, 12-hour light/dark cycle) and maintained with NIH31 regular chow and tap water *ad libitum*. In all experiments, male C57BL/6NCr wild-type mice at 8–12 weeks of age were used. The MCD, CD, and methionine-deficient diet (MD) were purchased from Dyets Inc. (#518810, #518753, and #518896, respectively; Bethlehem, PA) and methionine- and choline-supplemented MCD diet (MCS, #518754; Dyets) was used as a control diet. The composition of these diets was shown in Supplementary Table 1. Before starting the experiments, the NIH31 chow was replaced with MCS for acclimatization. After acclimatization for three to five days, mice were moved to new cages and the respective diet was given. For the analysis of changes in serum metabolites and phenotypes after 3-day and 1-week MCD treatment, sera, epididymal white adipose tissues (eWAT), and liver tissues obtained from the mice reported previously [10] were used (n = 5/group). To assess the effect of MCD treatment on other WAT depots, mice were treated with MCS or MCD for one week (n = 7–8/group) and inguinal WAT, perirenal WAT, and mesentery were harvested. To examine the effect of methionine or choline supplementation on MCD treatment, mice were treated with (1) MCS with drinking deionized water; (2) MCD with drinking deionized water; (3) MCD with drinking deionized water containing choline bitartrate (30 mg/mL; Sigma-Aldrich, St. Louis, MO); and (4) MCD with drinking deionized water containing L-methionine (4 mg/mL; Sigma-Aldrich) for two weeks as described previously [10] (n = 5/group). To assess the influence of dietary deficiency of methionine or choline on the pathogenesis of MCD-NASH, mice were treated with MCD, CD, MD, or control MCS diet for two weeks (n = 4–7/group). For the fasting study, mice (n = 20) were treated with CD or MCS and randomly divided into two groups at two weeks after commencing the treatment. Mice in one group were fasted for 36 hours before killing and mice in the other group were continuously fed CD or MCS (n = 5/group). Throughout all experiments, mice were weighed and killed after a 6-hour fasting. Blood was collected using Serum Separator Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and centrifuged for 10 minutes at 8,000 *g* at 4 °C to isolate serum. Liver and WAT were isolated, weighed, and divided into the two parts. One part of the liver tissue and WAT from each mouse was immediately soaked in 10% neutral formalin for histological examination. Sera and the remaining liver and WAT were immediately frozen in liquid nitrogen and kept at −80 °C until use.

2.2. UPLC-ESI-QTOFMS analysis

Serum (6 μL) was diluted with 114 μL of 66% acetonitrile containing 5 μM of chlorpropamide as an internal standard and centrifuged twice at 18,000 *g* for 25 min at 4 °C

to eliminate precipitated proteins. The eluted sample (5 μL/injection) was introduced by electrospray ionization into the mass spectrometer [Q-TOF Premier® (Waters Corp., Milford, MA)] operating in either negative or positive electrospray ionization modes. All samples were analyzed in a randomized fashion to avoid complications due to artifacts related to injection order and changes in instrument efficiency. MassLynx software (Waters) was used to acquire the chromatogram and mass spectrometric data [10].

Centroided and integrated chromatographic mass data were processed by MarkerLynx (Waters) to generate a multivariate data matrix. Pareto-scaled MarkerLynx matrices including information on sample identity were analyzed by principal component analysis (PCA) and supervised orthogonal projection to latent structure (OPLS) analysis using SIMCA-P +12 (Umetrics, Kinnelon, NJ). The OPLS loading scatter S-plot was used to determine the ions that contributed significantly to the separation between MCD- and MCS-treated mice. The identity of ions with a correlation of 0.8 or higher to the model was further investigated. The identity of ions was confirmed by tandem mass spectrometry MS/MS fragmentation patterns.

2.3. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from liver tissue and WAT using a TRIzol Reagent (Invitrogen, Carlsbad, CA) and cDNA was generated from 1 μg RNA with a SuperScript II™ Reverse Transcriptase kit and random oligonucleotides (Invitrogen). Quantitative PCR was performed using SYBR green PCR master mix and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primer pairs were designed using qPrimerDepot (http://mouseprimerdepot.nci.nih.gov/) and listed as Supplementary Table 2. Measured mRNA levels were normalized to those of 18S ribosomal RNA and expressed as fold change relative to those of control mice.

2.4. Histological analysis

Small pieces of liver tissue and WAT were fixed in 10% neutral formalin, dehydrated by serial ethanol/xylene, and embedded in paraffin. Sections (4 μm thick) were stained by the hematoxylin and eosin method. At least three discontinuous sections were evaluated for each mouse.

2.5. Biochemical analysis

Serum levels of ALT, non-esterified fatty acid (NEFA), and glucose were measured with assay kits for ALT (Catachem, Bridgeport, CT), NEFA-HR(2) reagent (Wako Chemicals USA, Inc., Richmond, VA), and glucose (HK) (Sigma-Aldrich), respectively. Serum fibroblast growth factor 21 (FGF21) levels were assayed by use of the Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN). Hepatic TG contents were quantified using Wako TG kit (Wako Chemicals USA Inc.) as described elsewhere [13]. Measurement of protein concentrations was carried out using BSA™ protein assay kit (Thermo Scientific, Rockford, IL).

2.6. Immunoblot analysis

Approximately 40 mg of WAT was homogenized in RIPA buffer containing a proteinase inhibitor cocktail. The homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C to obtain lipid-free cytosolic extracts. Cytosolic extracts (30 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin or skim milk and incubated overnight with primary antibodies against hormone-sensitive lipase (HSL; Cell Signaling Technology, Inc., Danvers, MA, #4107, 1:1000 dilution), phosphorylated HSL (Cell Signaling, #4139, 1:1000 dilution), and adipose TG lipase (ATGL; Cell Signaling, #2439, 1:1000 dilution). The antibody against β-actin (abcam, Cambridge, MA. ab8227, 1:10000 dilution), was used to measure β-actin as the loading control. After three-times washing, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling, #7074, 1:3000 dilution) and scanned.

2.7. Statistical analysis

Quantitative data were expressed as mean ± standard deviation. Statistical analyses were performed using the two-tailed Student's *t*-test between the two groups and the one-way ANOVA test with Bonferroni's correction between the multiple groups, respectively. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Serum metabolomic analysis reveals significant increases in oleic and linoleic acids in early stage of MCD-NASH

Mice treated with MCD for three days and one week showed minimal to mild hepatic steatosis and inflammatory cell infiltration, and the mRNAs encoding F4/80 (EGF-like module containing, mucin-like, hormone receptor-like sequence 1, *Emr1*) and integrin alpha M (*Itgam*), which are expressed in macrophages and infiltrated inflammatory cells, were increased (Supplementary Fig. 1). Hepatic TG contents and serum ALT levels were increased even after a short 3-day MCD treatment [10]. To examine the initial metabolic changes associated with the occurrence of MCD-NASH, a metabolomic analysis was carried out using sera obtained from the mice treated with MCD or MCS for three days and one week. PCA of UPLC-ESI-QTOFMS negative mode data demonstrated clear discrimination between the MCD and MCS groups (Fig. 1A and Supplementary Fig. 2A). OPLS analysis also showed separation between the two groups (data not shown) that was further examined with S-plot loading scatter contribution analysis. This revealed three ions that were significantly increased after both 3-day and 1-week MCD treatment: linoleic acid (*m/s* 279.2325 with retention time 5.96 s), oleic acid (*m/s* 281.2484 with retention time 6.44 s), and 12-hydroxyeicosatetraenoic acid (12-HETE) (*m/s* 281.2484 with retention time 6.44 s) (Fig. 1B and Supplementary Fig. 2B). Because the increases in 12-HETE in sera of MCD-fed mice were reported previously [10], the present study focused on the changes in serum oleic and linoleic acids. The identities of oleic and linoleic acids were confirmed by MS/MS analysis (Supplementary Fig. 2C). Quantification of serum oleic and linoleic acid levels and measurement of NEFA concentrations revealed significant increases in these fatty acids (FA) in early stage of MCD-NASH (Fig. 1C and D).

3.2. Increases in serum oleic and linoleic acids are associated with enhanced FA release from WAT

Since WAT is a major source of FA in serum, early changes in WAT after MCD treatment were examined. Body weight and eWAT weight were markedly decreased (Fig. 1E) and histological appearance of eWAT demonstrated a reduction in adipocyte size with no apparent inflammatory cell infiltration and adipocyte browning (Fig. 1F). In order to assess the mechanism of changes in WAT caused by MCD treatment, the mRNA levels of genes associated with lipid metabolism were measured. The mRNAs encoding genes related to lipogenesis, such as fatty acid synthase (*Fasn*) and diacylglycerol O-acyltransferase 2 (*Dgat2*), were suppressed in mice fed a MCD (Fig. 2A). In addition, the mRNAs encoding ATGL (patatin-like phospholipase domain containing 2, *Pnpla2*) and carboxylesterase 3 (*Ces3*), involved in lipolysis, were significantly increased (Fig. 2B), and immunoblot analysis revealed marked increases in phosphorylated HSL in these mice (Fig. 2D). These results indicate acceleration of adipose lipolysis by MCD feeding. The lipolytic activity in WAT is enhanced by multiple factors, such as β-adrenergic receptor activation and inflammatory signaling, such as tumor necrosis factor α (TNFα) and interleukin 1β (Il-1β) [14]. The mRNAs encoding β2-adrenergic receptor (*Adrb2*) gene were up-regulated by MCD treatment, while *Tnf* and *Il1b* mRNA levels were not changed (Fig. 2C).

The effect of MCD treatment on other WAT depots was also examined. The weights of inguinal and perirenal WAT and mesentery were significantly decreased after 1-week MCD treatment (Fig. 3A) and histological appearance of inguinal and perirenal WAT revealed a marked reduction in adipocyte size (Fig. 3B). The mRNAs encoding *Fasn*, *Dgat2*, *Pnpla2*, *Ces3*, and *Adrb2* in inguinal and perirenal WAT showed similar changes to those in eWAT of the MCD-fed mice (Fig. 3C). Additionally, phosphorylation of HSL was significantly enhanced in inguinal and perirenal WAT of MCD-treated mice (Fig. 3D). Overall, these results clearly demonstrate that WAT lipolysis is markedly enhanced by MCD treatment and that serum FA increases in early stages of MCD-NASH are correlated with increased lipolytic activity in WAT.

3.3. Increases in serum oleic and linoleic acids and changes in WAT by MCD treatment are due to dietary methionine deficiency

To determine the contribution of dietary deprivation of methionine and choline to the changes in serum FA and WAT following MCD treatment, methionine or choline was supplemented to MCD-fed mice as described previously [10]. No significant differences in food consumption were noted between the groups (Supplementary Fig. 3A). Mice fed the MCD for two weeks showed histologically-confirmed steatohepatitis (Supplementary Fig. 3B and C) and similar changes in WAT and serum FA to 3-day and 1-week MCD feeding, i.e., decreased body weight, eWAT weight, and adipocyte size, increased serum oleic and linoleic acids, and increased *Ces3* and *Adrb2* mRNAs and HSL phosphorylation in WAT; (Fig. 4A–C, Fig. 5, and Supplementary Fig. 3D). These changes in serum FA and WAT following the MCD treatment were completely reversed by supplementation of methionine, but not choline (Fig. 4A–C, Fig. 5, and Supplementary Fig. 3D). Additionally, choline supplementation to MCD-fed mice, which mimics the condition of dietary methionine deficiency, markedly attenuated steatohepatitis, as evidenced by liver histology and hepatic

Emr1 and *Itgam* mRNA expression (Supplementary Fig. 3B and C), but it could not normalize the changes in serum FA and WAT (Fig. 4A–C). Hydrolysis of phospholipids and chylomicron/lipoproteins is another possible source of FA in serum [15]. Although the mRNAs encoding lecithin cholesterol acyltransferase (*Lcat*) and lipoprotein lipase (*Lpl*) were increased by 3-day and 1-week MCD treatment, respectively (Supplementary Fig. 4A), there was no meaningful association between the increases in serum oleic and linoleic acids and hepatic mRNAs encoding *Lcat* and *Lpl* (Supplementary Fig. 4B). Overall, these findings reveal a relationship between increased serum FA and enhanced WAT lipolysis upon MCD treatment. Also, these results suggest that the changes in serum FA and WAT following MCD treatment are due to dietary methionine deprivation, and not choline deprivation and hepatic/adipose inflammation.

3.4. MD treatment induces WAT lipolysis similar to MCD treatment

To verify whether dietary methionine deficiency induces WAT lipolysis and increases serum FA, mice were treated with MD or control MCS diet for two weeks. The body weight and eWAT mass were markedly decreased and serum NEFA concentrations were increased by MD treatment (Fig. 6A and B). Histological appearance of eWAT showed a marked decrease in adipocyte size without inflammatory cell infiltration (Fig. 6C). The levels of *Ces3* and *Adrb2* mRNAs and phosphorylated HSL were significantly increased in WAT of MD-fed mice (Fig. 6D and E). These results corroborate that dietary methionine deficiency enhances WAT lipolysis.

3.5. MD treatment decreases serum glucose concentrations and increases FGF21 levels

In addition to decreased body weight and WAT mass and increased serum FA, significant decreases in serum glucose and increases in hepatic *Fgf21* mRNA and serum FGF21 were noted in mice with MCD treatment that were all reversed by methionine supplementation (Fig. 4D and E). Similar changes in serum glucose and FGF21 were also detected in MD-treated mice (Fig. 6F and G). The mRNA encoding glucose transporter type 4 (*Glut4*, also called solute carrier family 2, member 4, *Slc2a4*) was increased in WAT of mice on MD (Fig. 6D) that might be associated with a reduction in serum glucose concentrations in these mice. These results suggest that MD treatment causes similar metabolic changes as the fasting response.

3.6. Role of MD in the progression of MCD-NASH

To understand the significance of dietary methionine deficiency and the following WAT lipolysis in the pathogenesis of MCD-NASH, liver phenotypes were compared between mice treated with MCS and MD, and between mice treated with CD and MCD for two weeks. CD treatment did not singularly affect body weight, but induced hepatic steatosis (Fig. 7A and B). MCD treatment markedly reduced body weight and serum glucose and increased serum FGF21 compared with CD treatment (Fig. 7A, C, and D). Although serum ALT and hepatic expression of *Tnf* and *Itgam* mRNAs were not increased by MD treatment compared with control MCS treatment, these parameters were significantly increased by MCD treatment compared with CD treatment (Fig. 7E and F, Supplementary Fig. 5). Consistent with serum ALT and hepatic *Tnf/Itgam* expression, hepatocyte degeneration and

inflammatory cell infiltration were not seen in the livers of MD-treated mice (Fig. 7G). Macrovesicular steatosis was seen in CD-fed mice, and hepatocyte damage and mild-to-moderate focal inflammation in addition to steatosis were detected in MCD-fed mice (Fig. 7G). Therefore, these results suggest that adding dietary methionine deficiency to CD-induced steatosis promotes liver injury that may lead to steatohepatitis. This unfavorable effect of additional dietary methionine deficiency under the CD-fed condition is not due to the direct hepatotoxicity caused by dietary methionine deprivation, but likely due to MD-induced metabolic alterations, such as increased adipose lipolysis and enhanced FA flux.

3.7. Role of CD in the steatogenesis of MCD-NASH

The impact of dietary choline deficiency on hepatic fat accumulation was also addressed. CD treatment did not affect the mRNAs encoding genes involved in *de novo* lipogenesis, such as ATP citrate lyase (*Acly*), acetyl-CoA carboxylase α (*Acaca*), and *Fasn* (Supplementary Fig. 6). Stearoyl-CoA desaturase 1 (*Scd1*) mRNA was decreased in CD- and MCD-fed mice. The mRNAs encoding genes associated with VLDL secretion, microsomal TG transfer protein (*Mttp*) and apolipoprotein B (*Apob*), were significantly altered by both treatments (Supplementary Fig. 6), supporting the previous findings that dietary choline deficiency disrupts VLDL secretion leading to hepatosteatosis [11].

3.8. Enhancing WAT lipolysis by fasting exacerbates liver injury in the presence of dietary choline deficiency

The addition of methionine deficiency to CD-induced steatosis aggravated liver injury and dietary methionine deficiency caused similar metabolic changes to fasting, i.e., acceleration of adipose lipolysis. Lastly, to explore the possibility that the MD-like metabolic alterations can exacerbate liver damage upon CD state, mice were treated with CD or MCS for two weeks and killed after a 36-hour fasting. A 36-hour fasting caused decreased body weight and serum glucose, increased serum FGF21 and oleic and linoleic acids in CD- and MCS-treated mice, which were similar to changes found in the MD-treated mice (Fig. 8A–D). Although the mRNAs encoding *Tnf* and *Itgam* did not increase (data not shown), serum ALT levels were significantly increased by fasting only in the CD-treated group (Fig. 8E). Similar to MD-treated mice, hepatocyte degeneration and inflammatory cell infiltration were not observed in fasted control mice (Fig. 8F). However, some inflammatory cells were seen in CD-treated mice with fasting (Fig. 8F). These results suggest a detrimental effect of MD-like metabolic changes, such as WAT lipolysis, to the progression of liver injury in the presence of dietary choline deficiency.

4. Discussion

The present study focused on serum metabolite changes in the early stages of MCD-NASH in order to explore the metabolic disruption associated with occurrence of NASH. Serum metabolomic analysis in mice treated with MCD for three days uncovered significant increases in oleic and linoleic acids early during the development of MCD-NASH, which was due to enhanced lipolysis in WAT. The changes in serum FA and WAT after the MCD treatment were due to dietary methionine deficiency. When MCD treatment was compared with CD treatment, the methionine deficiency-induced metabolic changes, such as WAT

lipolysis, aggravated liver injury. Collectively, these results indicate an important role for dietary methionine deficiency and WAT lipolysis in the development of MCD-NASH.

Serum FA levels are mainly influenced by dietary intake, storage and release from WAT, and hydrolysis of phospholipids and chylomicron/lipoproteins. Increased serum FA by MCD treatment were correlated with decreased body weight, WAT mass, and adipocyte size and elevated *Ces3* and *Adrb2* mRNA levels and phosphorylation of HSL in WAT, but not with changes in hepatic expression of *Lcat* and *Lpl* mRNAs, indicating a major contribution of WAT lipolysis to augmentation of serum FA. In humans having NAFLD, approximately 60% of hepatic TG was reported to originate from FA released from WAT [16]. Additionally, recent studies demonstrated that the overall TG hydrolysis activity was significantly increased in the WAT of MCD-treated mice, but not in the liver [17, 18], thus corroborating the present observations. Since storing FA as stable lipid droplets is a crucial role of WAT *in vivo*, inappropriate increases in lipolysis activity in WAT may be harmful to other FA-utilizing organs, such as liver. Notably, in steatotic hepatocytes under the CD state, FA overload from WAT causes excess reactive oxygen species (ROS) generation mainly in mitochondria. In response to the increased ROS, glutathione (GSH), a potent endogenous antioxidant, is consumed to detoxify ROS. Since methionine and choline can be precursors of GSH, addition of dietary methionine deficiency to the CD state might gradually reduce mitochondrial GSH and further lower the capacity to detoxify ROS [19]. These metabolic alterations enhance hepatic oxidative stress and sensitize steatotic hepatocytes to apoptosis and necrosis [20, 21], leading to the progression to steatohepatitis. It was reported that some patients developed NASH following massive weight loss e.g., after bariatric surgery [22] or pancreaticoduodenectomy [23]. The mechanism to develop NASH caused by malnutrition is considered to be similar to that of MCD-NASH. Furthermore, enhanced lipolysis in WAT and the predominance of FA use in the liver were also documented in humans having insulin resistance and NAFLD [16, 21]. The findings in the present study offer one of the potential mechanisms on how WAT lipolysis contributes to the development and progression of NASH/NAFLD.

Dietary methionine restriction is known to reduce adiposity [24]. This study strengthened the view that dietary methionine deficiency causes reduction in WAT mass, but the precise mechanism remains unclear. One possibility is induction of FGF21 in the liver. FGF21 is a hepatokine that improves insulin sensitivity and its overexpression in mice yields a lean phenotype, increased lipolysis in WAT, and resistance to diet-induced obesity [25]. FGF21 treatment to 3T3-L1 adipocytes was reported to induce lipolysis, suggesting the direct effect of FGF21 on WAT lipolysis [25]. Additionally, FGF21 was shown to stimulate glucose uptake in 3T3-L1 adipocytes [26] and FGF21 administration caused a marked decrease in plasma glucose in diabetic rhesus monkeys [27]. Therefore, dietary methionine deficiency can induce FGF21 synthesis in the liver, enhance glucose uptake in peripheral tissues, such as WAT, and lower serum glucose. As a consequence, sympathetic nerve activation occurs and WAT lipolysis is enhanced. Increased *Adrb2* and *Glut4* expression in WAT by MD treatment supports this hypothesis. Since elevated serum/liver FGF21 levels are reported in human NAFLD/NASH [28, 29], further studies are needed to clarify the contribution of FGF21 to the pathogenesis of NAFLD/NASH.

Another intriguing finding in the present study was up-regulation of *Ces3* (also termed triacylglycerol hydrolase, *Tgh*) in WAT by MCD and MD treatments. Although HSL and ATGL are the main lipolytic enzymes in WAT, multiple enzymes and several lipid droplet-coating proteins also regulate WAT lipolysis [14]. *Ces3* is one of the major lipases in adipocytes [30] and is induced by fasting in WAT [31]. The WAT of *Ces3/Tgh*-null mice showed a 50% decrease in total TG hydrolysis activity, a 70% increase in WAT mass, and significant decreases in serum FA and glycerol [32], suggesting a major contribution of *Ces3* in the regulation of overall lipolysis activity of WAT. Therefore, increased *Ces3* expression in WAT may, at least in part, be associated with enhanced WAT lipolysis by MCD and MD treatments. It may be of value to examine whether disruption of adipose *Ces3* can modulate the severity of MCD-NASH.

Although it is difficult to determine the relative contributions of HSL, ATGL, CES3, and other lipases/proteins to the MCD- and MD-induced lipolysis, the percentage of HSL and ATGL to the overall TG hydrolysis activity is estimated as approximately 70% and 20% in WAT of 2-week MCD-treated mice, respectively, based on the results of a recent study [17]. Phosphorylation of HSL and up-regulation of *Adrb2* appeared even after a 3-day MCD treatment, thus indicating significant sympathetic nerve activation and HSL phosphorylation in early stages of MCD-NASH. On the contrary, the levels of ATGL protein seemed to be gradually increased. This observation is partially in agreement with the published finding that ATGL protein was increased in *ob/ob* mice treated with MCD for five weeks [18]. The present study could not assess the levels of phosphorylated ATGL and actual ATGL activity in 3-day or 1-week MCD-fed mice, while the previous study indicated up-regulation of ATGL activity in WAT of 2-week MCD-fed mice [17].

The finding in this study that WAT lipolysis may aggravate liver injury in the presence of CD-induced steatosis seems to be not entirely consistent with a previous observation that ATGL plays a protective role in MCD-induced hepatic steatosis and inflammation [17]. However, ATGL is a major lipase not only in WAT but also in liver that regulates peroxisome proliferator-activated receptor α (PPARα) signaling. In fact, suppression of ATGL in hepatocytes by injecting adenovirus encoding ATGL short hairpin RNA to mice led to marked down-regulation of PPARα and its downstream targets [33]. Since PPARα plays a critical role in attenuating hepatic TG accumulation and inflammation [34], hepatic ATGL may protect hepatocytes from lipotoxicity and inflammatory signaling through modulating PPARα. To address the precise role of adipose ATGL in the context of several liver diseases, mice with adipocyte-specific disruption of ATGL may be of value to examine in future studies.

In the present study, MD treatment did not cause liver injury, which was different from a previous report [35]. Although the reason of this discrepancy was unclear, MD treatment did increase hepatic mRNA levels of DNA-damage inducible transcript 3, a typical marker of endoplasmic reticulum (ER) stress. Since persistent ER stress can lead to mitochondrial damage and hepatocyte apoptosis [36], longer MD treatment might induce liver damage to some degrees.

Recently, others reported significant increases in linoleic acid in the serum and liver of *ob/ob* mice treated with the MCD for five weeks [18], a finding that is in agreement with the results in the present study. Linoleic acid is an unsaturated and not saturated, such as palmitic and stearic acids. Although direct toxicity of palmitic acid was shown in isolated hepatocytes [36], linoleic acid is auto-oxidized *in vivo* and can produce several lipid peroxides, such as 4-hydroxynonenal and malondialdehyde [37]. Increased release of linoleic acid from WAT and its influx into the liver may further amplify lipotoxicity and trigger the progression to NASH.

In conclusion, the present study revealed an important role of WAT lipolysis in the development of MCD-NASH. These findings provide the possibility that modulating WAT lipolysis may be a novel therapeutic target of NASH/NAFLD. Elucidating changes in WAT functions in patients having NASH might lead to further comprehensive understanding of the pathogenesis of NASH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Acaca	acetyl-coenzyme A carboxylase alpha
Acly	ATP citrate lyase
Adrb	β-adrenergic receptor
ALT	alanine aminotransferase
Apob	apolipoprotein B
ATGL	adipose triglyceride lipase
CD	choline-deficient diet
Ces3	carboxylesterase 3
Dgat	diacylglycerol O-acyltransferase
Emr1	EGF-like module containing, mucin-like, hormone receptor-like sequence 1
ER	endoplasmic reticulum
eWAT	epididymal white adipose tissue

FA	fatty acid
Fasn	fatty acid synthase
FGF	fibroblast growth factor
Glut4	glucose transporter type 4
GSH	glutathione
HETE	hydroxyeicosatetraenoic acid
HSL	hormone-sensitive lipase
IL	interleukin
Itgam	integrin alpha M
Lcat	lecithin cholesterol acyltransferase
Lpl	lipoprotein lipase
MCD	methionine- and choline-deficient diet
MCS	methionine- and choline-supplemented MCD diet
MD	methionine-deficient diet
Mttp	microsomal triglyceride transfer protein
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NEFA	non-esterified fatty acid
OPLS	orthogonal projection to latent structure
PCA	principal component analysis
Pnpla2	patatin-like phospholipase domain containing 2
PPAR	peroxisome proliferator-activated receptor
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
Scd1	stearoyl-coenzyme A desaturase 1
SS	simple steatosis
TG	triglyceride
TNF	tumor necrosis factor
UPLC-ESI-QTOFMS	ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry
VLDL	very-low-density lipoprotein
WAT	white adipose tissue

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Highlights

- Significant increases in fatty acids were observed in early stages of MCD-NASH.
- The increases in fatty acids were due to enhanced adipose lipolysis.
- These changes were derived from dietary methionine deficiency, but not hepatitis.
- We report an important role for adipose lipolysis in the development of MCD-NASH.

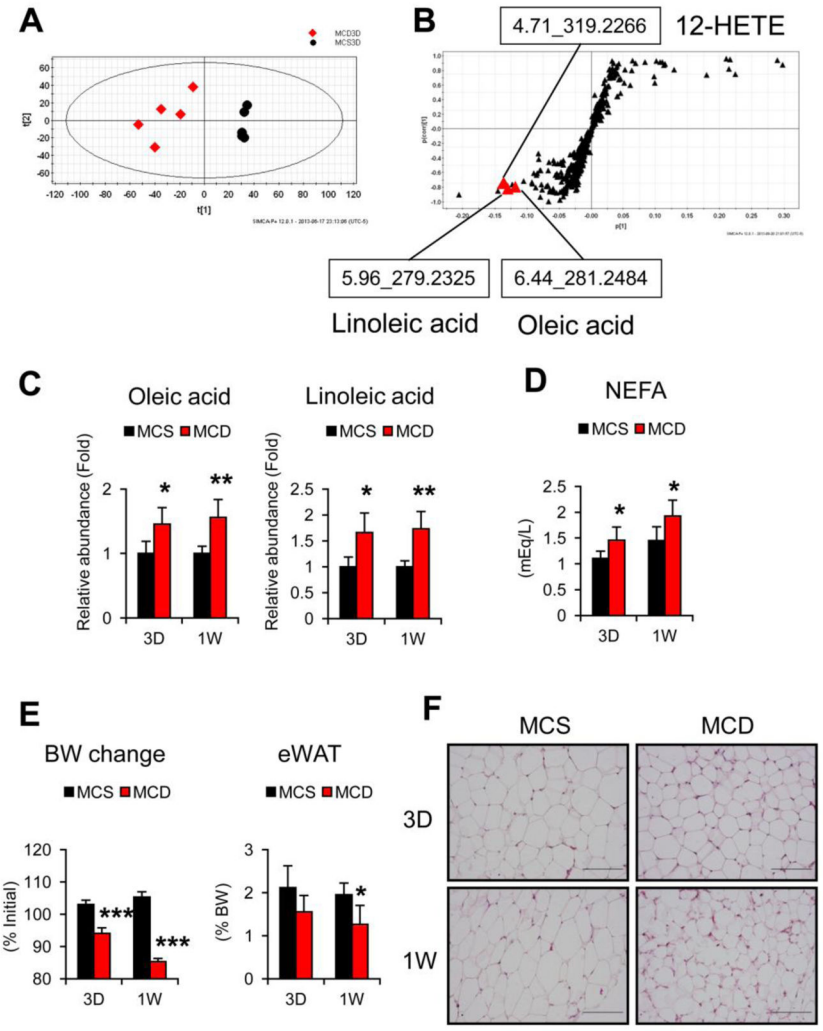

Fig. 1. Serum metabolomic analysis reveals significant increases in oleic and linoleic acids in early stage of MCD-NASH

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were fed a methionine- and choline-deficient diet (MCD) or control methionine- and choline-supplemented MCD diet (MCS) for three days or one week (n = 5/group).

(A) PCA of serum metabolites between mice treated with 3-day MCD (red diamond) and MCS (black circle).

(B) S-plot of OPLA analysis using the same data as (A). Retention time and molecular mass were indicated.

(C) Serum levels of oleic and linoleic acids. Values were normalized to those of MCS-treated mice in each time point and were expressed as relative abundance.

(D) Serum levels of NEFA.

(E) Body weight (BW) change and epididymal WAT (eWAT) weight. BW was measured just prior to killing and BW changes were expressed as the percentage relative to BW just before commencing the MCD or MCS treatment.

(F) Histology of epididymal WAT. Hematoxylin and eosin staining, Bar = 100 μm. Statistical analysis was performed using the Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. MCS-treated mice in the same time point.

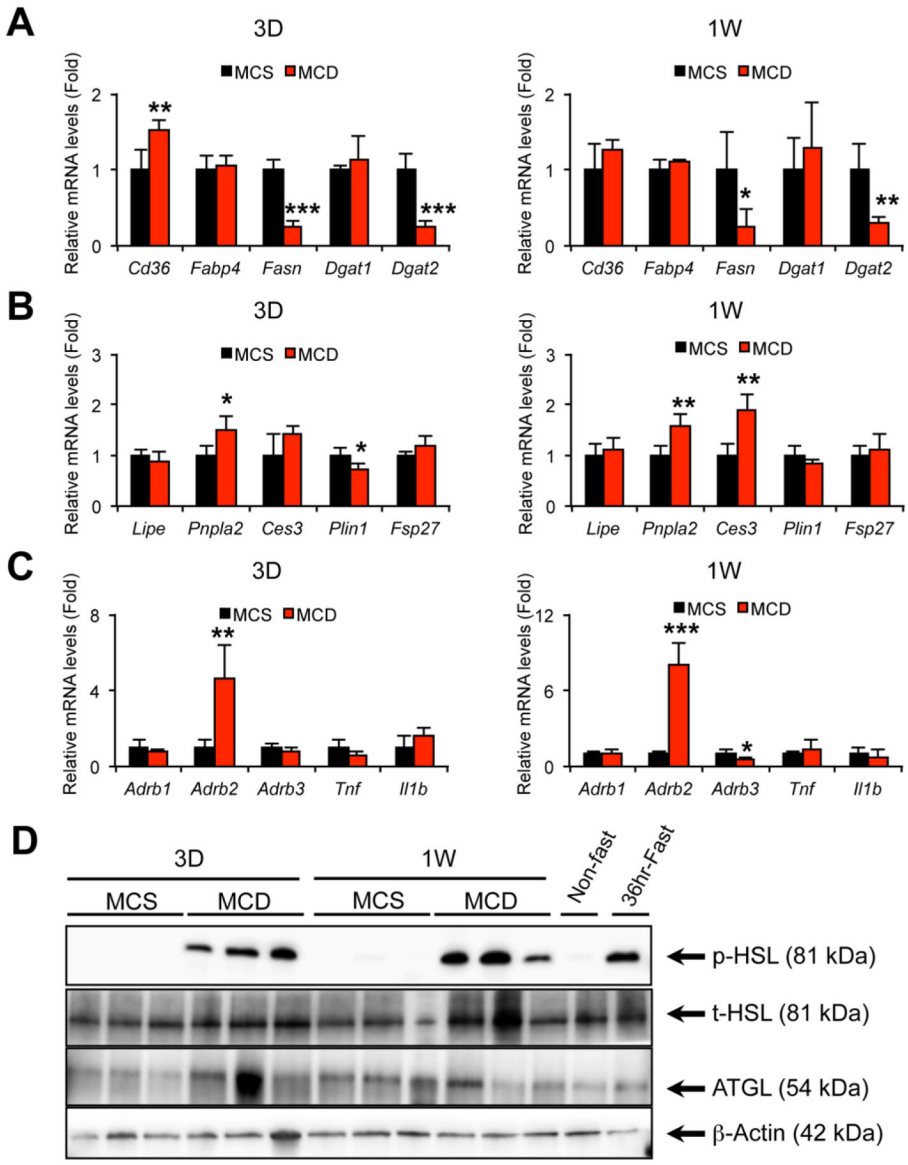

Fig. 2. Quantitative PCR analysis of the genes associated with lipid metabolism and immunoblot analysis of lipolytic enzymes in the epididymal WAT of mice treated with MCD for 3 days or 1 week

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were treated with MCS or MCD for three days or one week (n = 5/group) and epididymal WAT was subjected to qPCR and immunoblot analyses.

(A–C) qPCR analysis. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice. Statistical analysis was performed using the Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. MCS-treated mice in the same time point. Full terms of the gene names were listed in Supplementary Table 2.

(D) Immunoblot analysis of phosphorylated and total hormone-sensitive lipase (p-HSL and t-HSL, respectively) and adipose triglyceride lipase (ATGL). Cytosolic extracts of WAT (30 μg of protein) were loaded in each well. The band of β-actin was used as a loading control.

Epididymal WAT isolated from a 36 hour-fasted mouse was used for detecting the true position of HSL and ATGL bands.

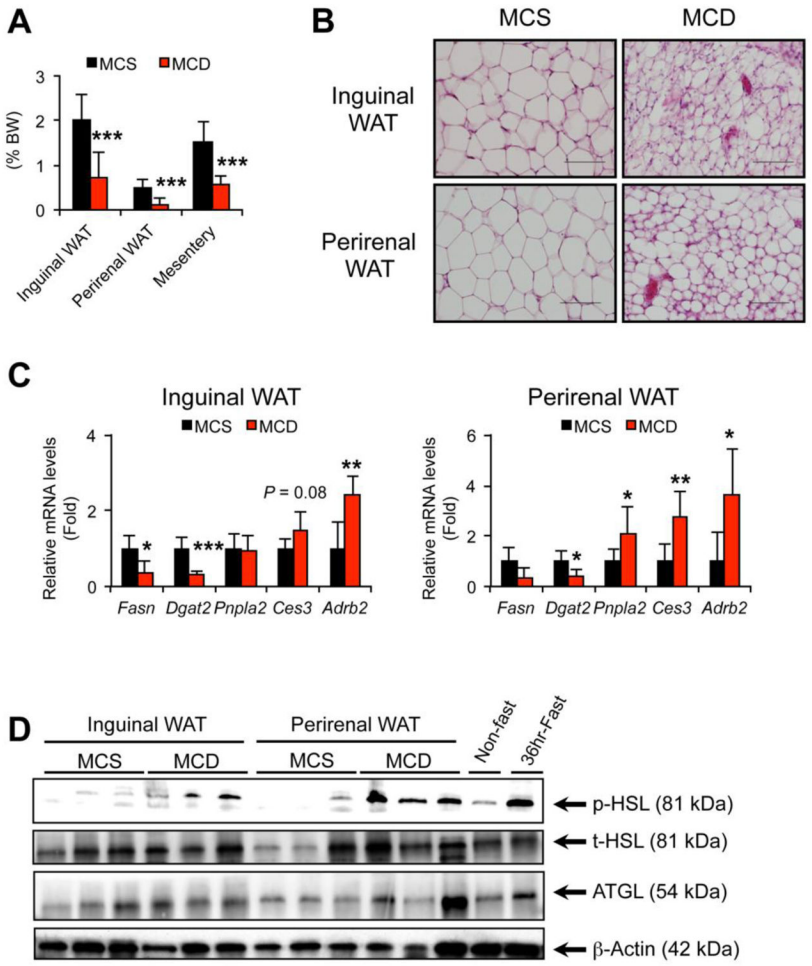

Fig. 3. The effect of MCD treatment on other WAT depots

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were treated with MCS or MCD for one week (n = 7–8/group) and inguinal WAT, perirenal WAT, and mesentery were harvested.

(A) The weights of inguinal and perirenal WAT and mesentery.

(B) Histology of inguinal and perirenal WAT. Hematoxylin and eosin staining, Bar = 100 μm.

(C) qPCR analysis. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice. Statistical analysis was performed using the Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. MCS-treated mice.

(D) Immunoblot analysis of p-HSL, t-HSL, and ATGL. Cytosolic extracts of WAT (30 μg of protein) were loaded in each well. The band of β-actin was used as a loading control.

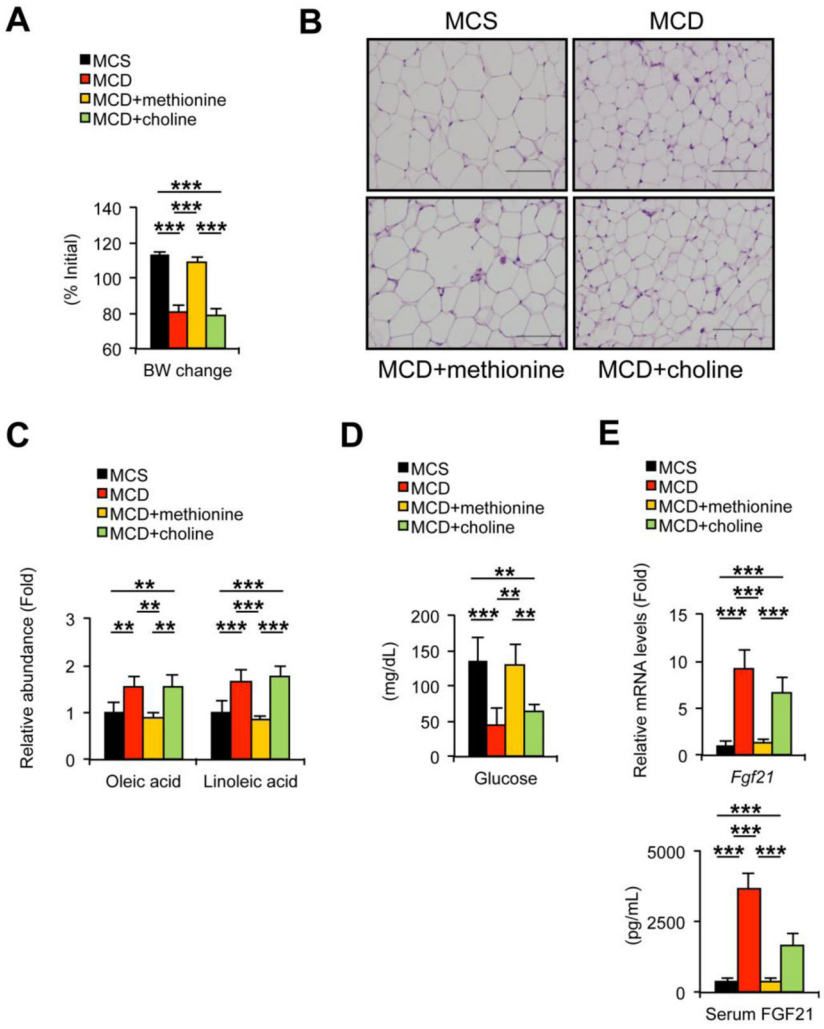

Fig. 4. Increases in serum oleic and linoleic acids and changes in WAT by MCD treatment are reversed by methionine supplementation

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were treated with MCS with drinking deionized water, MCD with drinking deionized water, MCD with drinking deionized water containing L-methionine (4 mg/mL, MCD+methionine), or MCD with drinking deionized water containing choline bitartrate (30 mg/mL, MCD+choline) for two weeks (n = 5/group) and serum, liver, and epididymal WAT were collected.

(A) Body weight (BW) change. Values were expressed as the percentage relative to BW just before commencing the MCD or MCS treatment.

(B) Histology of epididymal WAT. Hematoxylin and eosin staining, Bar = 100 μm.

(C) Serum levels of oleic and linoleic acids. Values were normalized to those of MCS-treated mice and were expressed as relative abundance.

(D) Serum glucose concentrations.

(E) Hepatic *Fgf21* mRNA levels and serum FGF21 concentrations. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice.

Statistical analysis was performed using the one-way ANOVA test with Bonferroni's correction. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

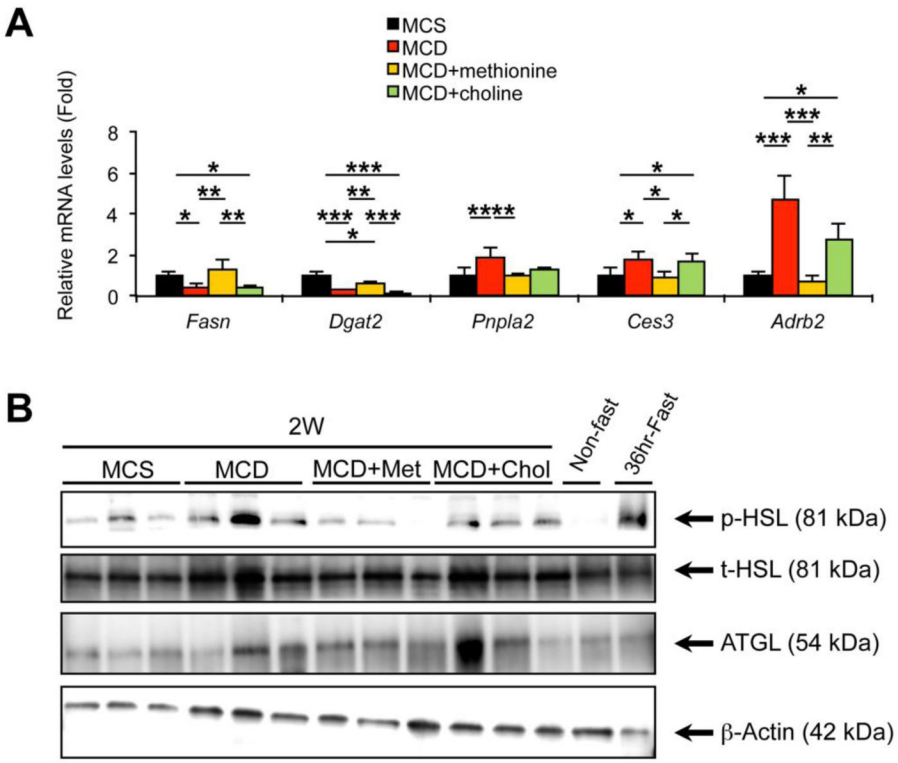

Fig. 5. The effect of supplementation of methionine or choline on gene/protein expression in the WAT of MCD-treated mice

Epididymal WAT isolated from mice shown in Fig. 4 was subjected to qPCR and immunoblot analyses.

(A) qPCR analysis of the indicated genes. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice. Statistical analysis was performed using the one-way ANOVA test with Bonferroni's correction. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

(B) Immunoblot analysis of p-HSL, t-HSL, and ATGL. Cytosolic extracts of WAT (30 μg of protein) were loaded in each well. The band of β-actin was used as a loading control. Met, methionine; Chol, choline.

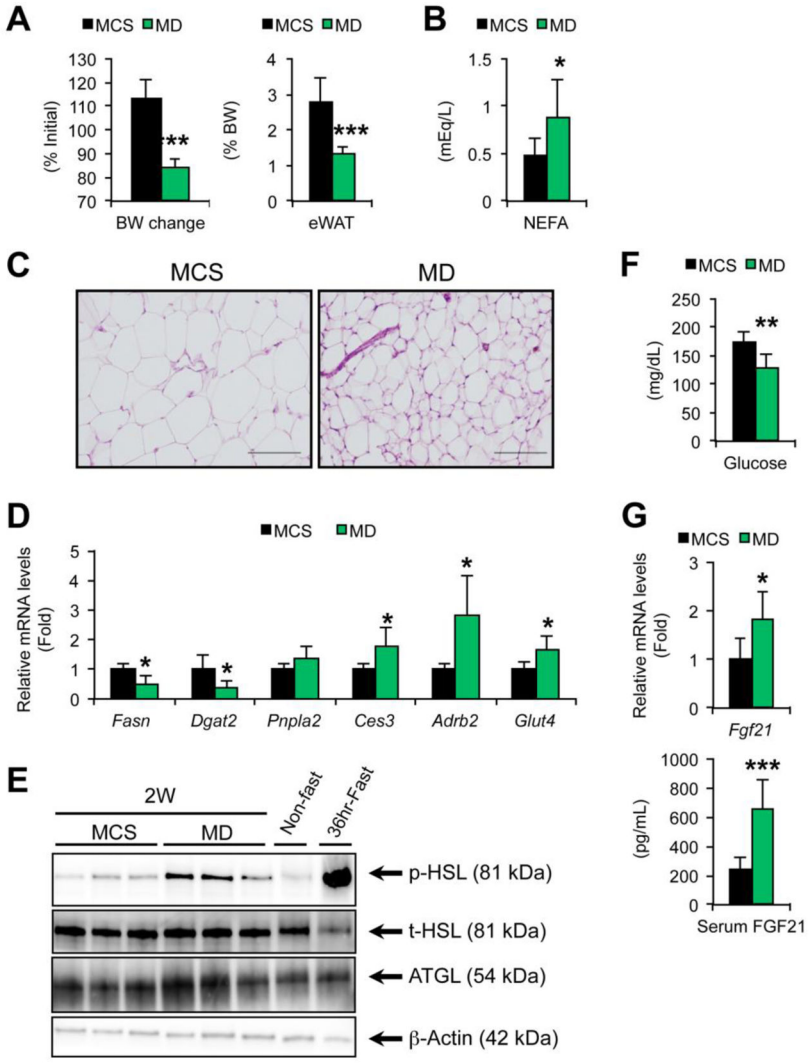

Fig. 6. MD treatment causes similar metabolic alterations to MCD treatment

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were treated with methionine-deficient diet (MD) or control MCS diet for two weeks (n = 7/group) and serum, liver, and epididymal WAT (eWAT) were harvested.

(A) Body weight (BW) change and eWAT weight. BW change values were expressed as the percentage relative to BW just before commencing the MD or MCS treatment.

(B) Serum NEFA levels.

(C) Histology of eWAT. Hematoxylin and eosin staining, Bar = 100 μm.

(D) The mRNA levels of indicated genes in eWAT. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice.

(E) Immunoblot analysis of p-HSL, t-HSL, and ATGL. Cytosolic extracts of WAT (30 μg of protein) were loaded in each well. The band of β-actin was used as a loading control.

(F) Serum glucose concentrations.

(G) Hepatic *Fgf21* mRNA levels and serum FGF21 concentrations. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice.

Statistical analysis was performed using the Student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. MCS-treated mice.

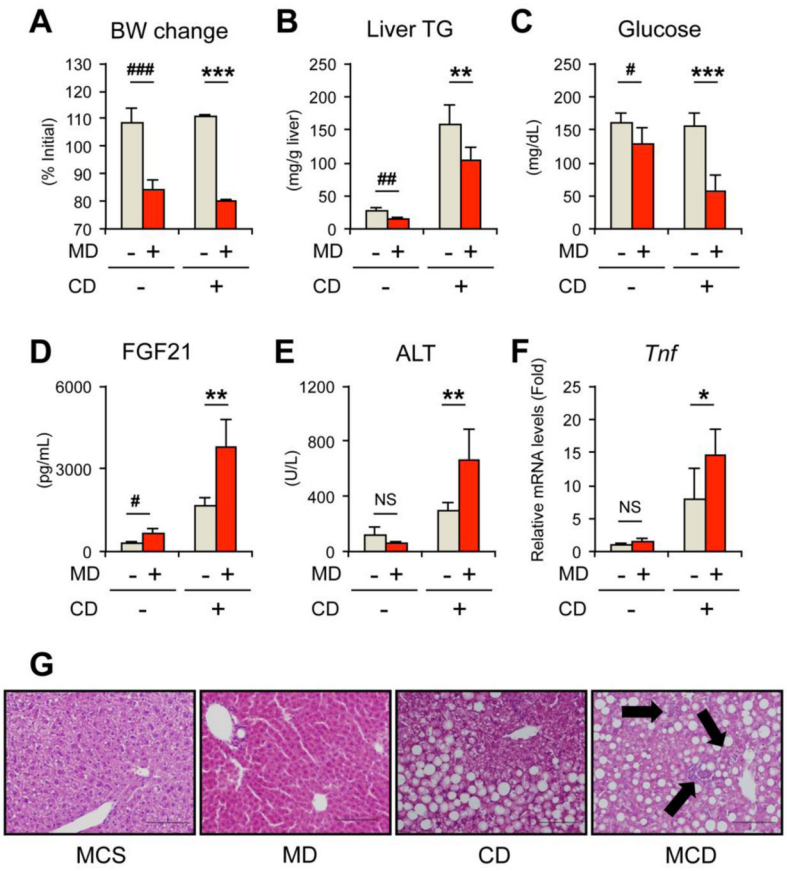

Fig. 7. The effect of dietary methionine deficiency on liver pathology

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were treated with MCD (designated as MD+ CD+), choline-deficient diet (CD, designated as MD− CD+), methionine-deficient diet (MD, designated as MD+ CD−), or control MCS diet (designated as MD− CD−) for two weeks (n = 4–7/group) and serum and liver were collected.

(A) Body weight (BW) change. Values were expressed as the percentage relative to BW just before commencing the MCD, CD, MD, or MCS treatment.

(B) Liver TG contents.

(C–E) Serum levels of glucose (C), FGF21 (D), and ALT (E).

(F) Hepatic *Tnf* mRNA levels. The mRNA levels were normalized to those of 18S ribosomal mRNA and subsequently normalized to those of MCS-treated mice.

(G) Representative liver histology. Hematoxylin and eosin staining, Bar = 100 μm. Arrows in MCD photograph indicate inflammatory foci.

Statistical analysis was performed using the Student's *t*-test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. MCS-treated mice (MD− CD−); $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. CD-treated mice (MD− CD+); NS, not significant.

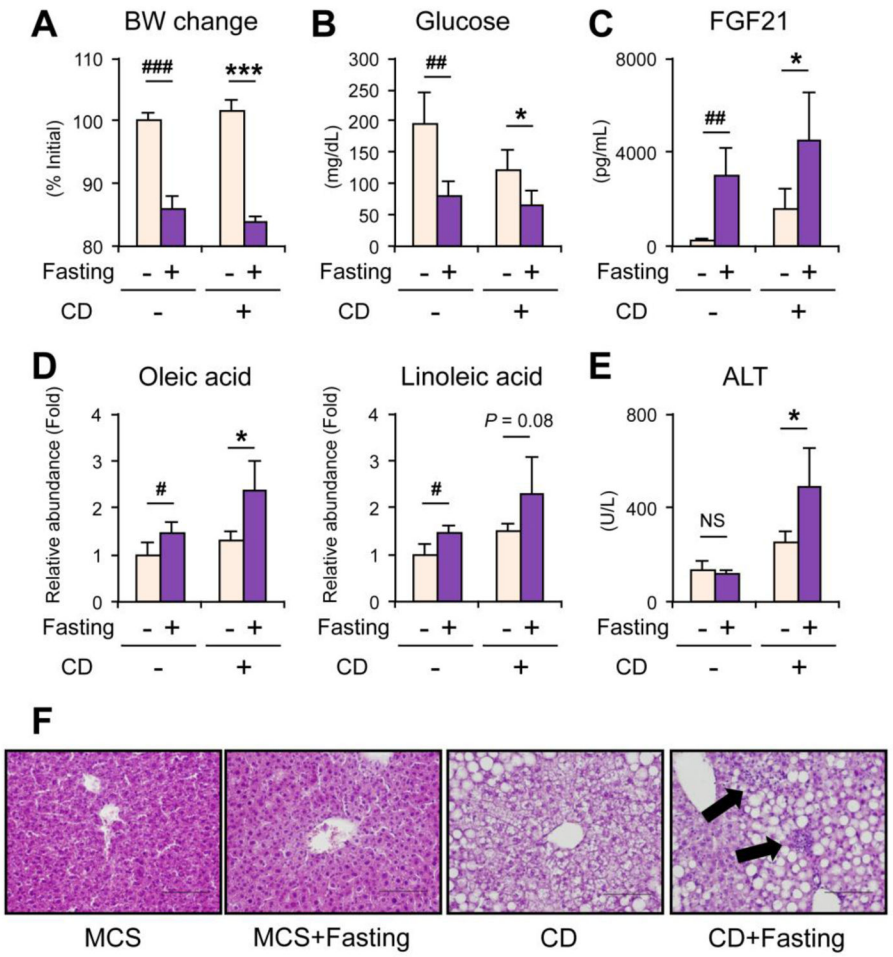

Fig. 8. Enhanced WAT lipolysis by 36-hour fasting exacerbates liver injury in the presence of dietary choline deficiency

Male C57BL/6NCr wild-type mice at 8–12 weeks of age were treated with choline-deficient diet (CD, designated as CD+) or control diet (MCS, designated as CD−) for two weeks and randomly divided into fasting or non-fasting group (n = 5/group). Mice in one group were fasted for 36 hours before killing (fasting+) and mice in the other group were continuously fed CD or MCS (fasting−). Serum and liver were harvested.

(A) Body weight (BW) change. Values were expressed as the percentage relative to BW at 36 hours before killing.

(B–E) Serum levels of glucose (B), FGF21 (C), oleic and linoleic acids (D), and ALT (E). Serum oleic and linoleic acid levels were normalized to those of non-fasted MCS-treated mice and were expressed as relative abundance.

(F) Representative liver histology. Hematoxylin and eosin staining, Bar = 100 μm. Arrows indicate focal inflammation.

Statistical analysis was performed using the Student's *t*-test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. non-fasted MCS-treated mice; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. non-fasted CD-treated mice; NS, not significant.