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Enhanced expression of Nrf2 in mice attenuates the fatty liver produced by a methionine- and choline-deficient diet[☆]

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Abstract

Oxidative stress has been proposed as an important promoter of the progression of fatty liver diseases. The current study investigates the potential functions of the Nrf2–Keap1 signaling pathway, an important hepatic oxidative stress sensor, in a rodent fatty liver model. Mice with no (Nrf2-null), normal (wild type, WT), and enhanced (Keap1 knockdown, K1-kd) expression of Nrf2 were fed a methionine- and choline-deficient (MCD) diet or a control diet for 5 days. Compared to WT mice, the MCD diet-caused hepatosteatosis was more severe in the Nrf2-null mice and less in the K1-kd mice. The Nrf2-null mice had lower hepatic glutathione and exhibited more lipid peroxidation, whereas the K1-kd mice had the highest amount of glutathione in the liver and developed the least lipid peroxidation among the three genotypes fed the MCD diet. The Nrf2 signaling pathway was activated by the MCD diet, and the Nrf2-targeted cytoprotective genes Nqo1 and Gsta1/2 were induced in WT and even more in K1-kd mice. In addition, Nrf2-null mice on both control and MCD diets exhibited altered expression profiles of fatty acid metabolism genes, indicating Nrf2 may influence lipid metabolism in liver. For example, mRNA levels of long chain fatty acid translocase CD36 and the endocrine hormone Fgf21 were higher in livers of Nrf2-null mice and lower in the K1-kd mice than WT mice fed the MCD diet. Taken together, these observations indicate that Nrf2 could decelerate the onset of fatty livers caused by the MCD diet by increasing hepatic antioxidant and detoxification capabilities.

Keywords

Nrf2; Fatty liver disease; MCD diet; Mouse

Introduction

Nonalcoholic fatty liver disease (NAFLD), defined as the accumulation of fat in the liver in the absence of alcohol consumption, is now becoming the most common cause of liver disease in Western countries (Angulo, 2002). NAFLD correlates strongly with obesity, type II diabetes, and other components of the “metabolic syndrome” (Chitturi et al., 2002; Marchesini et al., 2003). Although the exact mechanisms for the development of NAFLD remain unknown (Marra et al., 2008), extensive lipid accumulation and lipid peroxidation-induced oxidative stress are considered major factors causing cytotoxicity and exacerbated hepatopathy (Tilg and Diehl, 2000). However, the function of the hepatic antioxidative system during disturbed lipid metabolism has not been characterized.

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The nuclear factor erythroid 2-related factor 2 (Nrf2) serves as a master regulator of a cellular defense system against oxidative stress. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) with Cullin 3-base E3 ligase, by which Nrf2 protein is ubiquitinated and targeted for proteasome degradation (Cullinan et al., 2004). Upon exposure to electrophiles and reactive oxygen species (ROS), the sequestration complex breaks down and the dissociated Nrf2 translocates into the nucleus, where it binds to *cis*-acting antioxidant-responsive elements (AREs) and promotes the transcription of a broad range of cytoprotective genes (Itoh et al., 1997; Kensler et al., 2007). These genes encode enzymes that provide antioxidants, such as heme oxygenase-1 (Prester et al., 1995), synthesize reduced glutathione (GSH), such as glutamate cysteine ligase catalytic subunit (Gclc) (Moinova and Mulcahy, 1999), detoxify xenobiotics and electrophiles, such as glutathione *S*-transferases (GSTs) (Hayes et al., 2005) and NAD(P)H:quinone oxidoreductase 1 (Nqo1) (Aleksunes et al., 2006), as well as multidrug response transporters that efflux toxic metabolites (Maher et al., 2007).

Activation of Nrf2 plays a crucial role in regulating the cytoprotective responses to a variety of drugs, toxicants, and cellular stresses. Compared to wild-type (WT) mice, mice that lack Nrf2 (Nrf2-null) cannot induce these cytoprotective genes upon oxidative insult (Aleksunes and Manautou, 2007). Nrf2-null mice are extremely susceptible to exogenous challenges that result from oxidative and electrophilic stresses, such as acute liver toxicity induced by acetaminophen (Enomoto et al., 2001). In contrast, mice bearing a hepatocyte-specific knockdown of Keap1 (K1-kd) have constitutively high levels of Nrf2 in the nucleus and exhibit striking resistance to acetaminophen-induced hepatotoxicity (Okawa et al., 2006).

Nrf2 has also garnered attention in nutrition-related liver diseases. Previous studies showed that feeding mice with diets containing ethanol (Lamle et al., 2008) or a high concentration of fat (Tanaka et al., 2008) resulted in more severe liver injuries in Nrf2-null mice than wild-type mice. More recently, a global analysis of mouse hepatic gene expression revealed that both genetic and pharmacologic activation of Nrf2 resulted in induction of a larger cluster of genes associated with lipid metabolism than xenobiotic detoxification (Yates et al., 2009). Thus, the Nrf2–Keap1 signaling pathway appears to play an important role in nutrient metabolism, and potentially during the development of fatty liver diseases.

The purpose of the current study is to determine the effects of Nrf2 activation and the enhanced expression of Nrf2 target genes on the development of experimental fatty liver disease. To fulfill this task, mice with genetically low to high levels of active Nrf2 protein (Nrf2-null, WT, and K1-kd) were fed a methionine- and choline-deficient (MCD) diet for 5 days to induce simple fatty liver. Physiological changes and related hepatic gene expression in the three genotypes were then characterized. The ultimate goal of this investigation is to come to a better understanding of the importance of the cellular antioxidant system, especially the Nrf2 signaling pathway, in maintaining liver health.

Materials and methods

Animals and experimental design

Nrf2-null and Keap1-knockdown (K1-kd) mice were backcrossed 8 generations into C57BL/6J background (> 99%) as described (Reisman et al., 2009b) and used for experiments at 7 weeks of age. As controls, age-matched male C57BL/6 (WT) mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and acclimated for a minimum of 1 week in a temperature/humidity-controlled facility. WT, Nrf2-null, and K1-kd mice were fed either a control diet (#518754) or a methionine- and choline-deficient (MCD; #518810) diet for 5 days. The diets were purchased from Dyets Inc. (Bethlehem, PA). Both diets contained similar nutrients (14.2% protein, 15% fat, 3.09% ash, and 5%

fiber), except that methionine and choline were not included in the MCD diet, whereas 1.70 g/kg methionine and 14.48 g/kg choline bitartrate were provided in the control diet. All animals were fed *ad libitum* and had free access to water. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

After feeding the diets for 5 days, blood samples were collected and centrifuged (6,000×g) at 4 °C for 15 min to collect serum. Livers were removed and weighed. Two portions of the left lobe of each liver were either embedded in optimum cutting temperature compound or fixed in 10% formalin for subsequent microscopic analysis. The remainder of the liver was snap frozen in liquid nitrogen and stored at –80 °C until use.

Histopathology

Formalin-fixed liver sections (5 µm) were stained with hematoxylin and eosin (H&E) to evaluate severity of histological changes. Frozen liver sections (8 µm) were stained with Oil Red O and counterstained with hematoxylin for lipid content determination.

Blood sample profiles

Blood biochemistry analyses were done by Physicians Reference Laboratory (Kansas City, KS).

Liver triglyceride concentrations

Liver lipids were extracted as described (McGrath and Elliott, 1990). Triglyceride concentrations were determined using the Triglyceride (GPO) (Liquid) Reagent Set (Pointe Scientific Inc., Canton, MI) following the manufacturer's instructions.

Lipid peroxidation in livers

Lipid peroxidation in liver was monitored by quantifying thiobarbituric acid reactive substances (TBARS). About 25 mg of liver was dounce homogenized in 2 volumes of ice-cold PBS, and TBARS were determined via the OXltek TBARS kit (ZeptoMetrix, Buffalo, NY). Sample homogenates, as well as malondialdehyde (MDA) standards, were incubated with sodium dodecyl sulfate and thiobarbituric acid at 95 °C for 1 h. After incubation, samples were cooled on ice and centrifuged at 1,800×g for 15 min. Supernatants (200 µl) were transferred to a 96-well plate and quantified at 532 nm. MDA equivalents were expressed as nmol of MDA equivalents per gram liver.

Glutathione concentrations in livers

Approximately 20 mg of liver was homogenized in 5 volumes of KCl buffer (1.15%) and processed as described (Chen et al., 2008). The concentrations of glutathione (GSH) were determined by UPLC-MS/MS (Chen et al., 2008).

RNA extraction

Total liver RNA was prepared using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. RNA was dissolved in diethyl pyrocarbonate-treated deionized water, and RNA concentrations were quantified spectrophotometrically at 260 nm.

Branched DNA (bDNA) signal amplification analysis

The mRNA expression of major genes involved in the Nrf2–Keap1 signaling pathway and fatty acid metabolism was quantified by bDNA assay (Panomics/Affymetrix Inc., Fremont, CA) with individual liver RNA samples. Oligonucleotide probe sets (capture, label, and blocker probes) for each gene were designed for gene sequences accessed from GenBank as described previously (Cheng et al., 2005; Petrick and Klaassen, 2007; Reisman et al., 2009a). All probes were designed with a melting temperature of 63 °C. Five micrograms of total liver RNA from individual mice was added to a 96-well plate that was pre-loaded with capture reagent and the respective probe set. After overnight hybridization at 53 °C, hybridizations with amplifier, label, and substrate were subsequently carried out according to the manufacturer's protocol. Luminescence was quantified with a Synergy 2 Multi-Detection Microplate Reader interfaced with Gen5 Reader Control and Data Analysis software (Biotek, Winoosky, VT). Messenger RNA was presented as relative light units (RLU) per 5 µg of total RNA.

Western blotting

Nuclear and cytosolic extracts were prepared with the NE-PER nuclear extraction kit according to the manufacturer's manual (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Equal amounts of nuclear protein were used to determine Nrf2 protein concentrations. Cytosolic protein extracts were loaded to determine Nqo1 and Gclc protein. Nrf2 antibody (sc-13032) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Nqo1 (Ab2346), β -actin (Ab8227) and TATA binding protein (TBP; Ab818) were purchased from Abcam (Cambridge, MA). Primary antibody for Gclc was provided by Terry Kavanagh, University of Washington, Seattle, WA. Protein–antibody complexes were detected using an enhanced chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ). Molecular weights of Nrf2 (dimer), Nqo1, Gclc, and β -actin proteins were approximately 110, 25, 75, and 45 kDa, respectively. Individual blot densities were quantified by Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA). The individual blot densities of Nqo1 and Gclc proteins were normalized to the loading control β -actin. Nrf2 protein was normalized to nuclear protein loading control TBP.

Statistical analysis

All data were analyzed using two-way analysis of variance (ANOVA) with genotype and diet as main factors, followed by Student–Newman–Keuls comparisons to assess the differences between groups ($p \leq 0.05$).

Results

Body weight and liver weight changes after MCD feeding

After 5 days, mice fed the control diet had similar body weights regardless of genotype, whereas the MCD diet resulted in significant weight loss in Nrf2-null and WT mice, but not in K1-kd mice (Fig. 1A). In mice fed the control diet, liver weights were lowest in Nrf2-null (0.95 g and 4% of the body weight) and highest in K1-kd mice (1.52 g and 6.1% of the body weight) (Fig. 1B). The average liver weight of WT mice fed the control diet was 1.33 g and contributed to 5.5% of the body weight. The MCD diet decreased liver weight and liver–body weight ratio in both WT and K1-kd mice but not in Nrf2-null mice (Fig. 1C).

Effects of MCD diet on liver pathology

Hematoxylin and eosin (H&E) staining revealed swollen hepatocytes with moderate microvesicular steatosis in WT and K1-kd mice, and macrovesicular steatosis and lobular inflammation in Nrf2-null mice (Fig. 2A). Oil Red O staining showed that feeding the MCD diet for 5 days resulted in increased hepatic lipid deposition, which was most apparent in Nrf2-null mice and least in the K1-kd mice (Fig. 2B). The increases in liver triglyceride content paralleled the histological changes (Fig. 2C). Although the MCD diet increased fat content in livers of all genotypes, K1-kd mice accumulated less triglycerides than Nrf2-null and WT mice (29 vs. 43 and 41 mg/g liver).

Blood biochemistry

Blood concentrations of triglycerides and very low density lipoproteins (VLDL) were both decreased in all genotypes fed the MCD diet, although statistical significance was only achieved in WT and K1-kd mice (Fig. 3). Increases of serum bilirubin concentrations (mainly direct bilirubin), aspartate transaminase (AST), and alanine transaminase (ALT) activities were observed in Nrf2-null mice fed the MCD diet. In K1-kd mice, only serum ALT was altered by the MCD diet.

Hepatic glutathione concentration and lipid peroxidation

In mice fed the control diet, hepatic glutathione (GSH) concentrations correlated with Nrf2 expression in that Nrf2-null mice had lower and K1-kd exhibited higher GSH content than WT mice (Fig. 4). After feeding the MCD diet for 5 days, hepatic GSH concentrations were increased in all genotypes; however, statistical significance was only observed in Nrf2-null mice.

Malondialdehyde (MDA) is a reactive and toxic product of reactions between reactive oxygen species and polyunsaturated lipids. MDA is commonly used as a biomarker to quantify lipid peroxidation. As shown in Fig. 4, the MCD diet increased MDA 103% in Nrf2-null mouse livers, 68% in WT mice, but not in K1-kd mouse livers. Thus, lipid peroxidation induced by the MCD diet correlated inversely with Nrf2 activity in mouse liver.

Nrf2–Keap1 signaling pathway

Messenger RNA expression of several Nrf2 target genes is shown in Fig. 5. Basal expression of Gclc and Gsta1/2 was not different between genotypes. However, expression of the prototypical Nrf2 target gene NAD (P)H:quinone oxidoreductase 1 (Nqo1) was higher in the Nrf2-enhanced K1-kd mice. Furthermore, Nqo1 mRNA was induced by the MCD diet in an Nrf2-dependent manner, with the highest Nqo1 expression in K1-kd mice. The MCD diet also increased Gclc mRNA expression in WT and K1-kd mice and Gsta1/2 mRNA in K1-kd mice. No induction of Nqo1, Gclc, and Gsta1/2 was observed in Nrf2-null mice fed the MCD diet.

Nrf2 activation was further verified by Western blotting. As shown in Fig. 6, accumulation of Nrf2 protein in the nucleus was increased by the MCD diet in mouse livers expressing Nrf2. The Nrf2 protein band was absent in the Nrf2-null mouse livers. Furthermore, the hepatic expression of Nqo1 protein correlated with Nrf2 expression (Fig. 7). Nqo1 was highest in the cytosol of K1-kd mice and was minimally detected in Nrf2-null mice. The basal expression of Gclc protein was also influenced by Nrf2 expression, as the highest Gclc protein was detected in K1-kd mouse livers and lowest in Nrf2-null mice. However, the MCD diet increased protein expression of Gclc in both Nrf2-null and WT mice, indicating Nrf2-independent regulation of Gclc expression.

Lipid metabolism-related gene expression

Interestingly, several transcription factors and enzymes involved in fatty acid metabolism were differently expressed in the three genotypes fed control and MCD diets. The expression of nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α) was similar in the three genotypes fed the control diet (Fig. 8). The MCD diet increased PPAR α mRNA expression in WT and K1-kd mouse livers. Cytochrome P450 enzyme Cyp4a14 is a PPAR α target gene and plays an important role in catalyzing lipid oxidation in microsomes (Leclercq et al., 2000). The expression of Cyp4a14 was high in Nrf2-null and low in K1-kd mice fed the control diet, whereas the MCD diet-induced Cyp4a14 mRNA to a similar amount in all three genotypes. When the control diet was fed, mRNAs of sterol regulatory element binding protein 1c (SREBP-1c) and fatty acid synthase (Fas) were expressed higher in Nrf2-null mice than in the WT and K1-kd mice. The MCD diet did not change the mRNA expression of SREBP-1c or Fas in WT or K1-kd mice but decreased their expression in Nrf2-null mice to similar values as in the other two genotypes. The hepatic expression of the fatty acid translocase CD36 (cluster of differentiation 36) and fibroblast growth factor 21 (Fgf21) was increased by the MCD diet in all genotypes. However, both CD36 and Fgf21 were expressed highest in Nrf2-null mouse livers, and lowest in the livers of K1-kd mice on either the control or the MCD diet.

Discussion

The lipogenic methionine- and choline-deficient diet promotes intrahepatic lipid accumulation in rodents by increasing hepatocyte fatty acid uptake and decreasing VLDL-triglyceride secretion (Rinella et al., 2008). Elevated liver triglycerides and serum ALT were previously reported in A/J mice after feeding an MCD diet for 3 days, and minimal focal lobular inflammation was observed after 1 week (Sahai et al., 2004). In the present study, mice expressing various amounts of Nrf2 were fed the MCD diet for 5 days. Decreased circulating VLDL and triglycerides were observed in all three genotypes fed the MCD diet (Fig. 3), indicating impaired hepatic export of triglycerides by the MCD diet, which has been demonstrated previously (Rinella et al., 2008). The hepatosteatosis was more severe in Nrf2-null mice and much less in the K1-kd mice after feeding the MCD diet for 5 days (Fig. 2). Furthermore, increased serum bilirubin, AST, and ALT were observed in the Nrf2-null mice fed the MCD diet (Fig. 3), indicating more hepatocellular injury in these mice. Thus, the deficiency of Nrf2 increases the susceptibility of mice to the MCD diet-induced hepatosteatosis and liver injury.

Increased expression of Nrf2 has been reported in livers of mice fed a high-fat diet for 12 weeks (Kim et al., 2004). In the current study, the Nrf2 signaling pathway was activated by the MCD diet as indicated by increased nuclear accumulation of Nrf2 proteins and the induction of Nrf2-target genes (Figs. 5–7). Compared to WT mice, the induction of genes encoding cytoprotective enzymes Nqo1 and Gsta1/2 by the MCD diet was enhanced in K1-kd mice but lost in the Nrf2-null mice. Thus, the inability to induce these detoxification enzymes renders the Nrf2-null mice more susceptible to the MCD diet-induced hepatocellular injury. In contrast, the enhanced expression of these enzymes protects the K1-kd mice.

An important mechanism by which Nrf2 helps to maintain cellular redox status is through regulating of glutathione synthesis (Reddy et al., 2007). As shown in Fig. 4, K1-kd mice have the highest hepatic GSH concentrations and were protected from the lipid peroxidation induced by the MCD diet. In comparison, Nrf2-null mice had lower cellular GSH concentrations, and had the most MDA among the three genotypes fed the MCD diet. Nrf2 transcriptionally regulates Gclc, the catalytic subunit of the rate limiting enzyme of GSH biosynthesis (Chan et al., 2001). The MCD diet-induced Gclc mRNA in WT and K1-kd mice, but not in Nrf2-null mice. However, Gclc protein and GSH concentrations were

increased in Nrf2-null mice fed the MCD diet, indicating regulatory mechanisms in addition to Nrf2 that modulate GSH concentrations in liver. Maintaining high levels of cellular GSH appears to be important in the Nrf2-mediated protection from the MCD diet-induced liver injury in mice.

The mechanism by which the MCD diet activates Nrf2 is not known. However, a likely explanation is that the Nrf2–Keap1 complex senses the oxidized environment caused by the MCD diet. The ω -oxidation of lipids, which is partially catalyzed by Cyp4a, might be a major contributor to the oxidative stress in the MCD model (Leclercq et al., 2000). In the current study, Cyp4a mRNA was induced by the MCD diet in all three genotypes (Fig. 8). The increased expression of Cyp4a enhances hepatic lipid turnover but also increases the production of hepatic lipoperoxides (Ip et al., 2003). The oxidized products of fatty acids could also directly trigger the Nrf2 signaling pathway. This is supported by an *in vitro* study where oxidized n-3 fatty acids were shown to react with Keap1 and induce the expression of Nrf2-target genes (Gao et al., 2007).

To examine the MCD diet-induced changes in hepatic lipid metabolism in mice expressing various amounts of Nrf2, we quantified the mRNA expression of several key transcription factors and their prototypical target genes involved in lipid metabolism in the liver. PPAR α , the key regulator of fatty acid oxidation in hepatocytes, and its target gene Cyp4a14 were both induced by the MCD diet (Fig. 8). However, genotype differences were not observed in these genes, suggesting that fatty acid oxidation was increased in the MCD diet-fed mouse livers but was not influenced by the expression of Nrf2. SREBP-1c is a lipogenic transcription factor, and its target gene, Fas, encodes a key enzyme in *de novo* fatty acid synthesis. Previously, both SREBP-1c and Fas were reported to be decreased in wild-type mice fed the MCD diet for 3 weeks (Rizki et al., 2006). The reduction of fatty acid synthesis could be considered a protective response against hepatosteatosis. In the current study, both SREBP-1c and Fas were expressed at higher levels in Nrf2-null mice than WT and K1-kd mice when the control diet was fed; however, the MCD diet decreased both SREBP-1c and Fas mRNAs in the Nrf2-null mice but not in the other two genotypes. Thus, Nrf2 appears to decrease fatty acid biosynthesis in livers when mice were fed the control diet and helps to maintain homeostasis of fatty acids when the MCD diet was fed for a short time.

Furthermore, the mRNAs of CD36, a fatty acid transporter that facilitates the uptake of long chain fatty acids into liver (Ehehalt et al., 2008), and Fgf21, a liver-orientated endocrine hormone that stimulates lipolysis in white adipose tissue (Inagaki et al., 2007; Hotta et al., 2009), were induced by the MCD diet inversely to the expression of Nrf2. The MCD diet increases mRNA expression of CD36 the most in Nrf2-null mice and the least in K1-kd mice, which suggests higher free fatty acid uptake by hepatocytes in Nrf2-null and lower in K1-kd mice. Fgf21 mRNA was induced in Nrf2-null and WT mice after feeding the MCD diet for 5 days, which correlates with weight loss in these mice. However, both Fgf21 mRNA and weight loss induced by the MCD diet were attenuated in K1-kd mice. These data suggest that Fgf21 might mediate the weight loss induced by the MCD diet, and that the hepatic Fgf21 expression might reflect the progression of MCD diet-induced fatty livers.

In the MCD diet-fed K1-kd mice, the lack of changes of fatty acid metabolism-related genes could be due to less fat infiltration, a critical factor that influences fatty acid metabolism in liver (Marra et al., 2008). It is also intriguing to hypothesize that Nrf2 might influence the whole body energy distribution through its effects on hepatic genes, such as Fas, Fgf21, and CD36. Further experiments are required to verify these speculations.

Very recently, the role of Nrf2 was reported in mice fed the MCD diet for 2 weeks (Chowdhry et al., 2009), 3 weeks, and 6 weeks (Sugimoto et al., 2009). Consistent with the

current study, the previous studies also show that the absence of Nrf2 resulted in a lack of activation of detoxification and antioxidation systems as well as increased oxidative stress and hepatoseatosis in the MCD model. However, neither of these previous studies utilized the Keap1-knockdown mice as an Nrf2-enhanced model. Thus, the present study not only indicates that a loss of Nrf2 promotes but that an enhancement of Nrf2 attenuates the onset of the MCD diet-induced fatty livers.

In conclusion, the Nrf2 signaling pathway is activated in mouse livers by feeding an MCD diet. Enhancement of Nrf2 expression attenuates, and the deficiency of Nrf2 promotes the onset of fatty liver by the MCD diet. The protective effects of Nrf2 in the MCD dietary model might derive from (1) enhanced expression of antioxidant and detoxification genes that prevent intracellular lipid peroxidation, and (2) attenuation of the release of fatty acids from adipose tissue and reduction of hepatic uptake of fatty acids. However, more work is required to disclose whether and how Nrf2 influences fatty acid metabolism in liver. Taken together, the Nrf2 signaling pathway could be a therapeutic target for the prevention and treatment of fatty liver diseases.

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Abbreviations

CD36	cluster of differentiation 36
Fas	fatty acid synthase
Fgf21	fibroblast growth factor 21
Gclc	glutamate cysteine ligase catalytic subunit
GST	glutathione S-transferase
Keap1	Kelch-like ECH-associated protein 1
K1-kd	Keap1-knockdown
MCD	methionine and choline deficient

MDA	malondialdehyde
NAFLD	nonalcoholic fatty liver disease
Nqo1	NAD(P)H: quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
PPARα	peroxisome proliferator-activated receptor alpha
ROS	reactive oxygen species
SREBP-1c	sterol regulatory element binding protein 1c
TBP	TATA binding protein
WT	wild type

Fig. 1. Changes of body and liver weights after feeding the MCD diet for 5 days. (A) Body weight, (B) liver weight, and (C) liver-body weight ratio. Male Nrf2-null (Nrf2^{-/-}), wild-type (WT), and Keap1 knockdown (K1-kd) mice were fed a control diet (■) or a methionine- and choline-deficient (MCD) diet (□) for 5 days. Data are expressed as mean±SEM of 5 mice per group. *Statistically different from wild type on the same diet; †statistical difference caused by the MCD diet within each genotype. $p \leq 0.05$. The same illustrations are used in the following figures that are applicable.

Fig. 2. Changes in liver histopathology in Nrf2-null, WT, and K1-kd mice after feeding the MCD diet for 5 days. (A) Hematoxylin and eosin (H&E) staining. Arrow indicates infiltrated inflammatory cells. Genotype of each column is indicated above the picture and diet is shown on the left of the row. (B) Liver sections from each genotype stained with Oil Red O. The sections were examined by light microscopy, and the liver images are displayed at 200× original magnification. The horizontal bars inside each figure represent 100 μ m. (C) Hepatic triglyceride (TG) levels in the three genotypes after feeding the MCD diet for 5 days.

Fig. 3. Blood biochemistry of Nrf2-null, WT, and K1-kd mice after feeding the MCD diet for 5 days. TG, triglyceride; VLDL, very low density lipoprotein; AST, aspartate transaminase; and ALT, alanine transaminase.

Fig. 4. Liver antioxidant and lipid peroxidation capacities of Nrf2-null, WT, and K1-kd mice after feeding the MCD diet for 5 days. Glutathione (GSH) concentrations were quantified by UPLC-MS to indicate the antioxidant capability of livers from mice expressing various amount of Nrf2. Malondialdehyde (MDA) levels were quantified as a measure of lipid peroxidation in mouse livers.

Fig. 5.

Effects of feeding an MCD diet for 5 days on the mRNA expression of prototypical Nrf2-target genes. Nqo1, NAD(P)H:quinine oxidoreductase 1; Gcls, glutamate cysteine ligase catalytic subunit; Gsta1/2, glutathione *S*-transferases α 1/2. The mRNA expression of various genes were quantified by bDNA assay as described in Materials and Methods and presented as relative light units (RLU) per 5 μ g of total RNA.

Fig. 6.

Nuclear accumulation of Nrf2 protein after feeding the MCD diet for 5 days. (A) Twenty-five micrograms of nuclear protein from 2 (Nrf2-null) or 3 (WT and K1-kd) individual mice fed the control diet and 3 individual mice fed the MCD diet was used for Western blotting. Small arrows in the top panel indicates the specific Nrf2 protein band from the non-specific band below. (B) Protein blot densities were quantified by Quantity One 1-D Analysis Software. Nrf2 protein was normalized to nuclear protein loading control TATA binding protein (TBP).

Fig. 7. Protein expression of Nrf2 target genes Nqo1 and Gclc after feeding the MCD diet for 5 days. (A) Forty micrograms of cytosolic protein was loaded to determine Nqo1 and Gclc protein expression. (B) Nqo1 and Gclc proteins were normalized to the loading control β -actin. The sample numbers and protein quantification method are same as Fig. 6.

Fig. 8.

Messenger RNA expression of lipid metabolism genes in response to the MCD diet. PPAR α , peroxisome proliferator-activated receptor α ; Cyp4a14, cytochrome P450 4a14; SREBP-1c, sterol regulatory element binding protein 1c; Fas, fatty acid synthase; CD36, cluster of differentiation 36; Fgf21, fibroblast growth factor 21. The mRNA expression of various genes were quantified by bDNA assay as described in Materials and Methods and presented as relative light units (RLU) per 5 μ g of total RNA.