Hepatic Lipid Accumulation Induced by a High-fat Diet Is Regulated by Nrf2 Through Multiple Pathways

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Abstract

Background

Nuclear factor erythroid 2-related factor 2 (Nrf2) is reportedly involved in hepatic lipid metabolism, but the results are contradictory and the underlying mechanism thus remains unclear. Herein we focused on elucidating the effects of Nrf2 on hepatic adipogenesis and on determining the possible underlying mechanism. We established a metabolic associated fatty liver disease (MAFLD) model in high fat diet (HFD) fed Nrf2 knockout (Nrf2 KO) mice; further, a cell model of lipid accumulation was established using mouse primary hepatocytes (MPHs) treated with free fatty acids (FAs). Using these models, we investigated the relationship between Nrf2 and autophagy and its role in the development of MAFLD.

Results

We observed that Nrf2 expression levels were up-regulated in patients with MAFLD and diet-induced obese mice. Nrf2 deficiency led to hepatic lipid accumulation in vivo and in vitro, in addition to, promoting lipogenesis mainly by increasing SREBP-1 activity. Moreover, Nrf2 deficiency attenuated autophagic flux and inhibited the fusion of autophagosomes and lysosomes in vivo and in vitro. Weakened autophagy caused reduced lipolysis in the liver. Importantly, Chromatin immunoprecipitation-qPCR (ChIP-qPCR) and dual-luciferase assay results proved that Nrf2 bound to LAMP1 promoter and regulated its transcriptional activity. We accordingly report that Nrf2-LAMP1 interaction has an indispensable role in Nrf2-regulated hepatosteatosis.

Conclusions

These data collectively confirm that Nrf2 deficiency promotes hepatosteatosis by enhancing SREBP-1 activity and attenuating autophagy. To conclude, our data reveal a novel multi-pathway effect of Nrf2 on lipid metabolism in the liver, and we believe that multi-target intervention of Nrf2 signaling is a promising new strategy for the prevention and treatment of MAFLD.

Introduction

Metabolic associated fatty liver disease (MAFLD) has become a major health problem in humans and an important cause of cirrhosis and hepatocellular carcinoma. Its pathological alterations primarily include steatosis, lobular inflammation, and hepatocyte ballooning, eventually leading to the development of liver fibrosis and even hepatic cancer[1]. Approximately one-third of patients with MAFLD develop metabolic associated steatohepatitis (MASH). MAFLD is the result of multiple factors, including insulin resistance (IR), chronic oxidative stress, and lipotoxicity, as well as other conditions that promote hepatic inflammation and fibrosis[2–4]. Despite remarkable progress in understanding the molecular pathogenesis of MASH, to date, no effective drugs exist for its treatment. Therefore, it is vital to investigate the molecular mechanisms underlying MAFLD to ensure prevention and proper treatment.

Nuclear factor erythroid 2 related factor 2 (Nrf2) is a key transcription factor that regulates intracellular redox homeostasis[2]. Previous studies have shown that a reduction or enhancement in the activity of Nrf2 does not prevent diet-induced obesity and has less effect on lipid metabolism[5]. However, it has also been found that pharmacological activation of Nrf2 inhibits hepatic steatosis and reduces MASH and hepatic fibrosis in obese mice[6]. In addition, Nrf2 activation has been reported to inhibit hepatosteatosis and MASH on knockdown of its repressor Kelch-like ECH-associated protein-1 in methionine- and choline-deficient diet-fed mice[7]. Therefore, at present, the role of Nrf2 in the pathogenesis of MAFLD is contradictory, and accordingly, further studies are warranted.

Autophagy is a cellular process that degrades intracellular organelles and proteins. It targets invading microorganisms and involves abnormal protein accumulation to lysosomes degrade[8]. Functionally, autophagy is necessary to maintain the stability of the intracellular environment, and defects in autophagy are evidently related to the pathological process of some human diseases[9]. Previous studies have found that autophagy breaks down triglycerides (TG) via lipophagy and that the inhibition of autophagy in hepatocytes can cause hepatic lipid accumulation[10, 11]. In addition, genetic or pharmacological activation of autophagy has been found to reduce hepatosteatosis[12, 13]. However, the underlying mechanism via which autophagy regulates hepatic lipid accumulation remains debatable.

Some proteins play a pivotal role in the initiation of autophagy; for example, Atg3 and Atg7 down-regulation is reportedly related to autophagosomes formation[14]. Further, Atg7 overexpression has been found to alleviate MAFLD and IR in ob/ob mice and high-fat diet (HFD)-fed mice[15]. Some studies have also shown that the late stage of autophagy (such as autophagosome-lysosome fusion) plays a fundamental role in MAFLD occurrence and development, rather than in the initiation of autophagy[16]. In recent years, Nrf2 has been reported to regulate autophagy initiation through the signaling adapter p62-dependent pathway in response to oxidative damage via a positive feedback loop[17]. However, it remains unknown whether Nrf2 affects MAFLD occurrence and development by regulating autophagy in the liver.

Herein we established an MAFLD model in HFD-fed Nrf2 KO mice; further, a cell model of lipid accumulation was established using mouse primary hepatocytes (MPHs) treated with free fatty acids (FAs). Using these models, we investigated the relationship between Nrf2 and autophagy and its role in the development of MAFLD.

Materials And Methods

Mice and human hepatic tissue studies

Male 8-week-old C57BL/6J mice (wild-type, WT) were purchased from the Experimental Animal Center (Chongqing Medical University). 8-week-old male Nrf2 KO mice were purchased from Shanghai Bio-model and Ganismsci & Tech Develop Co., Ltd. (Shanghai, China). SREBP-1c knockout (SREBP-1c KO) mice were generously provided by Dr. Guan (Advanced Institute for Medical Sciences, Dalian Medical University, Liaoning, China). Nrf2 KO and SREBP-1c KO mice were generated as previously reported[18].

To establish MAFLD model induced by HFD, mice were fed a chow diet (NCD; 10% fat) or an HFD (60% fat; D12492; New Brunswick, NJ 08901 USA) for 12 weeks. Subsequently, mice were sacrificed and tissues were collected for further analyses. All animal protocols were approved by the Chongqing Medical University Animal Studies Committee and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Liver tissues were obtained *via* hepatic biopsy from six patients with MAFLD and six liver transplantation donors from the Department of Surgery, Xinqiao Hospital, Third Military Medical University. The protocols were reviewed and supported by the Ethics Committee of Xinqiao Hospital, Third Military Medical University, Chongqing China.

Gene Set Enrichment Analysis (GSEA)

Thirty-nine MAFLD samples from GSE89632 were divided into high and low expression groups, according to the median expression level of Nrf2. GSEA was then performed using GSEA v4.1.0 platform (<http://software.broadinstitute.org/gsea/index.jsp>)[19]. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets downloaded from the Molecular Signature Database served as reference. $P < 0.05$ was used for the identification of statistically significant GO and KEGG terms.

Biological Process (BP) Enrichment Analysis

Based on the GPL4134 platform, the(GSE35124)dataset was downloaded from the Gene Expression Omnibus (GEO) database. The raw expression data were preprocessed by the RMA algorithm in the affy package using the R statistical software. Differentially expressed genes (DEGs) were identified with the limma package[20]. BP enrichment analysis was performed with DAVID v6.8[21]. $P < 0.05$ determined statistical significance.

Cell culture and treatment

MPHs were isolated from 8-week-old WT, Nrf2 KO, and SREBP-1c KO mice, as previously described[18], and were cultured in DMEM/F12. HepG2 cells were cultured in DMEM with 10% FBS. On reaching 70% confluence, they were incubated with FA mixture (FAs, 1 mmol/L) at a 2:1 ratio of oleate/palmitate or BSA for 24 h, then treated with DMSO or ML385, an Nrf2 inhibitor, for 12 h (5uM, MCE, Shanghai, China).

Hematoxylin-Eosin (H&E) and Oil Red O staining

Paraffin-embedded livers from mice were processed, and paraffin sections (5 um) were stained with H&E. Frozen hepatic sections, HepG2 cells and MPHs were stained with 0.3% Oil Red O by standard procedures, as reported[22].

Immunohistochemistry (IHC)

Mice were anesthetized and perfused with 4% paraformaldehyde for 10 min. The liver was excised, transferred to 4% paraformaldehyde, and incubated at 4°C for 1 day. The samples were embedded by

paraffin and sliced. IHC to assess the expression levels of Nrf2, p62/SQSTM1 (p62), and LC3II were engaged as previously publication[23]. The primary antibodies used for IHC were anti-Nrf2, anti-p62, and anti-LC3 (1:400; ab62352, ab91526, ab229327; Abcam, Cambridge, England).

Autophagy flux measurement using adenovirus expressing mRFP-GFP-LC3 (Ad-mRFP-GFP -LC3)

HepG2 cells and MPHs from WT and Nrf2 KO mice were infected with Ad-*mRFP-GFP-LC3* (HanBio, Wuhan, China) as manufacturer's instructions. The confocal microscopy was used for observing the morphology of LC3 positive autophagosomes and autolysosomes. And the cells expressing LC3 were labeled with mRFP–GFP. Autophagosomes thus appeared as a yellow spot (RFP⁺GFP⁺), while autolysosomes appeared as a red spot (RFP⁺GFP⁻). The accumulation of LC3 spots was quantified to evaluate autophagy flux[24].

Determination of hepatic TG contents

Hepatic TG content was measured using a commercial kit (Applygen Technologies, Beijing, China) according to manufacturer instructions[25].

quantitative real-time PCR (qRT-PCR) and Western blotting

qRT-PCR was performed as previously reported[26]. Table S1 lists the forward and reverse primer sequences used for qRT-PCR, and Table S2 lists the primary antibodies used for Western blotting. qRT-PCR and Western blotting results were calibrated to β -actin or GAPDH[25].

Biochemical analysis

Serum total cholesterol (TC) and TG were enzymatically measured using an auto-analyzer. Serum alanine transaminase (ALT) and aspartate transaminase (AST) were measured using a commercial kit (Kebo biological industry co. LTD, Shandong, China) according to manufacturer instructions.

Luciferase reporter assay

Genomic PCR was performed to obtain DNA fragments containing the full-length mouse LAMP1 promoter and all deletion constructs (-1740/0, -1019/0, and - 851/0 bp) were generated as previously described[25]. Purified PCR products and pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA) were digested with XhoI and HindIII. Recombinant plasmids (pGL3-LAMP1-Luc) were verified by restriction endonuclease digestion and DNA sequencing. The primers used for the DNA fragments were listed in Table S3. Luciferase reporter assay was performed using a previously reported standard protocol[18]. Briefly, MPHs from WT and Nrf2 KO mice were transfected with reporter and Renilla-Luc plasmids (Promega, Madison, WI, USA) using Lipofectamine 3000 (Life Technologies). Luciferase activities were examined by a Dual luciferase kit (Promega) at 48 h post-transfection[25].

ChIP-qPCR

ChIP experiments were performed using MPHs of WT mice[27]. Briefly, 1% formaldehyde (EM grade; Thermo-Scientific) was used for crosslinking, a cell Lysis Buffer was used to lyse the cells, and ultrasound treatment was used to fragment DNA. The chromatin fraction was incubated with anti-Nrf2 (ab62352, Abcam, Cambridge, USA) or unspecific IgG (sc-2027, Santa Cruz Biotechnology Inc.). qPCR was conducted using SYBR Green Mix (Takara Bio, Otsu, Japan) for DNA fragment analysis. The amplicons were separated by electrophoresis on an agarose gel containing ethidium bromide and visualized under UV light. Specific primers were listed Table S4.

Statistics

The data were presented as the mean $\pm$ SEM. Statistical analysis was performed *via* Microsoft Excel and Prism software (Graphpad, La Jolla, CA). A two-way ANOVA with a least significant difference post-hoc test was used to compare the mean values between multiple groups, and an unpaired two-sided Student *t*-test was used for comparisons between two groups. $p < 0.05$ was considered statistically significant.

Results

Upregulation of hepatic Nrf2 in obese mice and patients with MAFLD

To determine the role of Nrf2 in obesity-related MAFLD, we first assessed the expression levels of Nrf2 and lipogenesis-related genes in patients with MAFLD. We observed that the expression levels of lipogenesis-related genes, including C/EBP α and β , Cidea and Ly6D, were significantly upregulated, suggestive of an increase in lipid synthesis in the liver (Fig. S1A). Importantly, the expression of Nrf2 at both mRNA and protein was markedly upregulated in the liver of patients with MAFLD, indicating its involvement in hepatic lipid deposition (Fig. 1A, B).

To further determine the role of Nrf2 in hepatosteatosis, we investigated hepatic Nrf2 expression levels in WT mice fed either NCD or HFD. As indicated in Fig. S1 B-D, histological and biochemical parameters confirmed MAFLD occurrence in HFD-fed mice. Next, we performed IHC, qRT-PCR and Western blots to investigate hepatic expression levels of Nrf2 mRNA and protein in WT mice fed either NCD or HFD, and we found that Nrf2 expression was markedly elevated both at mRNA and protein levels in HFD-fed mice (Fig. 1C, D). IHC also revealed that Nrf2 expression level was up-regulated in the liver from HFD-fed mice (Fig. 1E). Consistent with the results in mice and humans, the mRNA and protein expression levels of Nrf2 in FAs-treated MPHs were significantly up-regulated as compared to those in PBS-treated MPHs (Fig. 1F, G). These data demonstrate a key role of Nrf2 in MAFLD.

Deletion of Nrf2 facilitates hepatosteatosis induced by HFD

Considering the changes in Nrf2 mRNA and protein expression levels observed in obese mice and patients with MAFLD, we generated Nrf2 KO mice, and 8-week-old male WT and Nrf2 KO mice were fed either NCD or HFD to investigate the role of Nrf2 in hepatosteatosis (Fig. 2A). No significant changes were

observed in body weight and hepatic weight between NCD-fed Nrf2 KO and WT mice (Fig. 2B, D, E); however, HFD-fed Nrf2 KO mice showed significantly higher body weight and hepatic weight as compared with WT mice (Fig. 2B, D, E). Furthermore, at the macroscopic level, the liver showed a white appearance in HFD-fed Nrf2 KO mice (Fig. 2C). HE and Oil Red O staining revealed higher lipid accumulation in the liver of HFD-fed Nrf2 KO mice (Fig. 2F, G). Consistent with these changes, hepatic TG content, serum TG, CHO, ALT, and AST concentrations in Nrf2 KO mice fed an HFD were significantly increased than those in WT mice fed with the same diet (Fig. 2H-L). These data collectively suggest that hepatic Nrf2 has an important role in HFD-induced hepatosteatosis.

Deletion of Nrf2 enhances the expression of lipogenesis-related genes

To gain insights into the mechanism underlying the regulation of hepatic lipid metabolism by Nrf2, we analyzed genomic data pertaining to the liver tissues of patients with MAFLD from the GSE89632 dataset. GSEA revealed that Nrf2-related genes were mainly enriched in lipid metabolism (Fig. 3A). In addition, BP analysis of DEGs (GSE35124) revealed that Nrf2-related genes were most enriched in fatty acid synthesis (Fig. 3B).

To verify the relationship between Nrf2 and lipid metabolism, we investigated the expression levels of lipid-related genes in the liver of WT and Nrf2 KO mice fed either NCD or HFD. We observed that in comparison with HFD-fed WT mice, the protein expression levels of lipogenesis-related genes, including FAS, SCD-1, and Sterol regulatory element-binding protein 1c (SREBP-1c), were significantly upregulated in HFD-fed Nrf2 KO mice, while the phosphorylation level of ACC was significantly decreased (Fig. 3C). Further, oil red O staining showed that the lipid droplet contents of FAs-treated MPHs from Nrf2 KO mice and HepG2 cells treated with ML385 were significantly increased compared with those from WT mice and treated with DMSO (Fig. 3D, E). TG contents of MPHs from Nrf2 KO mice and HepG2 cells treated with ML385 were also higher (Fig. 3F, G). In these MPHs and HepG2 cells, the protein expression levels of lipogenesis-related genes, including SCD-1, FAS, and SREBP-1c, were significantly upregulated relative to control MPHs and HepG2 cells, while the phosphorylation level of ACC was significantly lower (Fig. 3H, I). Therefore, the results of our bioinformatics analyses and *in vivo* and *in vitro* studies validated that Nrf2 deficiency promotes lipogenesis and hepatic lipid accumulation.

SREBP-1 is crucial for Nrf2 to regulate adipogenesis

To further investigate how Nrf2 affects lipid metabolism, SREBP-1 KO mice were generated and MPHs were isolated, they were then incubated with BSA or FAs and treated with ML385 (an Nrf2 inhibitor) or DMSO (Fig. 4A). Further, the contents of TG and lipid droplets were significantly decreased in SREBP-1KO mice treated with ML385 and FAs compared with WT mice treated ML385 and FAs, but still higher than those in SREBP-1KO mice treated with DMSO and FAs (Fig. 4B, C). Importantly, the mRNA expression level of FAS and SCD-1 in ML385-treated MPHs from WT mice was significantly higher than that in DMSO-

treated MPHs, but no significant changes were observed in MPHs from SREBP-1 KO mice (Fig. 4D, E). These data confirmed that SREBP-1 plays a major role in Nrf2-mediated lipogenesis.

Hepatic autophagy signaling is involved in hepatosteatosis regulation by Nrf2

Autophagy is reportedly involved in lipid metabolism. To further investigate the mechanism by which Nrf2 mediates hepatic lipid metabolism, we performed GSEA using the liver tissues of patients with MAFLD from the GSE89632 dataset. We found that Nrf2-related genes were mainly enriched in the pathway related to lysosome function (Fig. 5A). Next, to confirm the findings of bioinformatics analysis, we examined the expression levels of LC3II (microtubule-associated protein 2 light chain 3) and SQSTM1/p62 in the liver of Nrf2 KO and WT mice fed either NCD or HFD[28]. The protein expression level of LC3II in the liver of HFD-fed Nrf2 KO mice was significantly downregulated in comparison with that in WT mice, which caused a significant decrease in the LC3II/LC3I ratio (Fig. 5B), suggesting an inhibition of autophagic flux. Furthermore, the protein expression of Atg7 in the liver of HFD-fed Nrf2 KO mice was significantly down-regulated in comparison with that in WT mice, while Atg3 was unchanged (Fig. 5B). Interestingly, we found that the protein expression level of SQSTM1/p62 (a substrate of autophagy) was markedly downregulated in the liver of Nrf2 KO mice than in that of WT mice (Fig. 5B). We speculate that the down-regulation of p62 may be due to the effect of Nrf2 knockout on p62 transcription as previous publications.[29] Moreover, in the liver of Nrf2 KO mice, IHC staining showed decreased expression levels of LC3II and SQSTM1/p62 (Fig. 5C). These results indicated that the lack of Nrf2 inhibited SQSTM1/p62 as well as autophagic flux.

Lysosomal degradation plays an important role in maintaining autophagic flux and recycling hepatic lipid droplets. We thus evaluated the expression levels of LAMP1 and LAMP2, which are lysosomal-associated membrane proteins,[30] in the liver of Nrf2 KO and WT mice. The protein expression level of LAMP1 was significantly downregulated in HFD-fed Nrf2 KO mice relative to that in WT mice, but the expression level of LAMP2 did not change (Fig. 5D), suggesting that Nrf2 deficiency causes lysosomal membrane dysfunction.

Effects of Nrf2 deficiency on lipid accumulation and autophagy in vitro

To further verify the relationship between Nrf2 deficiency and autophagy at the cellular level, MPHs from WT and Nrf2 KO mice were treated with FAs. As with *in vivo* studies, Western blotting showed that p62 protein expression level and LC3II densitometry in FAs-treated MPHs from Nrf2 KO mice and HepG2 cells treated with ML385 were significantly decreased compared with those in MPHs from WT mice and HepG2 cells treated with DMSO (Fig. 6A, C). LAMP1 and Atg7 protein levels also were lower in MPHs from Nrf2 KO mice and HepG2 cells treated with ML385 (Fig. 6B, C). To observe the effect of Nrf2 on autophagic flux more intuitively, we used a tandem labeled mRFP-GFP-LC3 reporter. HepG2 cells and MPHs from WT and Nrf2 KO mice were infected with Ad-mRFP-GFP-LC3 and LC3 punctate numbers were quantified for assessing the effect of Nrf2 inhibition or deficiency on autophagic flux. FAs-treated HepG2

cells and MPHs showed increased yellow fluorescent puncta and decreased red fluorescent puncta after 12h exposure. Cells from Nrf2 KO mice or treated with ML385 showed an even more significant reduction in red punctate (GFP⁻ and mRFP⁺), compared with MPHs from WT mice and HepG2 cells treated with DMSO, indicating that Nrf2 depletion further inhibited autophagic flux induced by FAs (Fig. 6D).

Nrf2 inhibits autophagy via the transcriptional regulation of LAMP1 promoter

One previous study has predicted LAMP1 region contains a putative ARE sequence.[31] To determine how Nrf2 regulates LAMP1 expression, we investigated the effect of Nrf2 on LAMP1 transcription in MPHs from WT mice. Figure 7A shows a diagram for predicting the binding site of Nrf2 and LAMP1 promoter. ChIP-qPCR results confirm that Nrf2 directly binds to the - 1740 bp to - 1726 bp region of LAMP1 promoter (Fig. 7B, C). Luciferase reporter assay results further indicated that a potential Nrf2-regulating site for LAMP1 promoter was located - 1740 bp to - 1726 bp upstream of the transcriptional start site (Fig. 7D).

Discussion

The pathogenesis of MAFLD is considered to be a complex, multifactorial process, and autophagy, chronic inflammation, and mitochondrial dysfunction are evidently involved in its occurrence and development[32]. Although Nrf2 has been reported to be involved in the pathogenesis of MAFLD, the results reported by previous studies are contradictory [5, 6, 33]. Therefore, the exact mechanism of Nrf2 in the occurrence and development of MAFLD remains unknown. Previous studies focused on the antioxidant effect of Nrf2 on lipid accumulation in the liver, while there were few reports about the Nrf2 regulation of lipogenesis in the liver[34]. In addition, previous studies were mostly descriptive and observational studies, lacking in-depth research, especially the molecular mechanism. In view of the particularity and complexity of MAFLD, it is urgent to further study the role of Nrf2 in the pathogenesis of MAFLD. Herein 1) our data confirmed that hepatic Nrf2 expression level was upregulated in patients with MAFLD and obese mice; 2) Nrf2 deficiency was observed to promote the development of hepatic lipid deposition when mice were fed HFD; 3) SREBP-1 activation was identified to be crucial for Nrf2 deficiency to promote fatty acid synthesis; 4) Nrf2 deficiency was found inhibit lipolysis by weakening autophagy; and finally, 5) Nrf2 deficiency was found to attenuate autophagy by inhibiting the transcription of LAMP1 promoter. Our findings thus reveal, in detail, the molecular mechanisms by which Nrf2 regulates hepatic lipid metabolism.

Herein our findings further confirmed that Nrf2 deficiency aggravated HFD-induced hepatosteatosis and FAs-induced intracellular lipid deposition. Consistent with our data, some previous studies have also found that Nrf2 deletion in hepatocytes resulted in a worse metabolic phenotype[33], resulting in higher liver tissue weight and the expression of inflammation-related factors in the liver[35]. In addition, a study reported that administering agonists activated Nrf2 to prevent obesity-induced by HFD in mice[36]. In contrast, another study reported that the lack of Nrf2 conferred protection from HFD-induced obesity in

mice[5]. Further, in genetically modified mice, Nrf2 has been observed to neither prevent the development of HFD-induced obesity nor show any involvement in the direct regulation of hepatic lipid metabolism[5, 37]. These contradictory results could be attributed to, for example, differences in experimental design, sources of fat in the diet, time of feeding the diet (i.e., HFD), gender, genetic background, and age of mice. Consequently, the mechanism by which Nrf2 deficiency leads to hepatic lipid accumulation is not completely understood. In this study, the genetic model was established using Nrf2 KO C57BL/6J mice to avoid the off-target effects of chemicals. All the mice were male, and to mimic the human diet, we fed the animals with 60% HFD or NCD for 12 weeks. Our data from patients with MAFLD further confirmed the relationship between Nrf2 and hepatosteatosis. Therefore, by controlling these factors, we believe that Nrf2 deficiency promotes diet-induced hepatic lipid deposition and obesity. Nevertheless, further studies are warranted to investigate the role of Nrf2 in hepatic lipid metabolism in more detail and to determine if Nrf2 interacts with other pathways that may lead to hepatic lipid accumulation.

SREBP-1, a transcription factor, regulates adipogenesis and glucose utilization in the liver, playing a pivotal role in fatty acid synthesis[18, 38]. *In vivo* and *in vitro* experiment results revealed that Nrf2 deficiency-mediated lipid accumulation was associated with significantly upregulated expression levels of genes encoding SREBP-1, FAS, and SCD-1. However, in MPHs from SREBP-1 KO mice, Nrf2 deficiency significantly reduced lipid accumulation. Thus, SREBP-1 appears to play a key role in Nrf2-mediated regulation of lipid metabolism. These results are consistent with previous studies[34, 36]. In addition, a previous study involving adipocytes reported that Nrf2 recruited SREBP-1 promoter and enhanced its transcription[39]. Importantly, we used SREBP-1 KO mice to provide unequivocal genetic evidence that Nrf2 can play a protective role in the initiation of hepatic lipid accumulation.

To obtain further insights into whether Nrf2 regulates lipid metabolism via multiple pathways, we performed GSEA using data from the GSE89632 database and found that Nrf2-related genes were mainly enriched in the pathway related to "lysosome." It is well known that autophagy decomposes intracellular lipids through lysosomal degradation[40]. Therefore, autophagy may be a candidate target for the Nrf2 regulation of hepatic lipid metabolism. Autophagy was recently reported to be associated with fat metabolism[41]. Autophagy dysfunction in the liver can inhibit fatty acid β -oxidation and TG output, causing hepatosteatosis *in vivo*[14, 42]. When autophagy is stimulated in the liver, hepatosteatosis improves[43]. In recent years, there are some lines of evidence indicating that Nrf2 is associated with autophagy in lung cancer cells and skeletal muscle[44–46]. In addition, Tang *et al.* reported that Nrf2 elevated the expression levels of genes related to autophagy in the brain of mice with age[47]. In contrast, Kitaoka *et al.* found that Nrf2 deficiency did not affect autophagy in the skeletal muscle of denervated mice[48]. We speculate that these contradictory results may be due to the different mechanisms of Nrf2 in different tissues and cells. Therefore, in the present study, we used Nrf2 KO mouse liver and primary hepatocytes to further explore the relationship between Nrf2 and autophagy. We found that in comparison with HFD-fed WT mice, autophagy was weakened in HFD-fed Nrf2 KO mice, as manifested by decreased LC3II/LC3I ratio and reduced p62, LAMP1, and Atg7 expression levels. Thus, our findings indicate that Nrf2 has a positive regulatory effect on HFD-induced autophagy *in vivo*. Although these

results are consistent with a previous study[47] but different from those of others[46, 48], we believe that Nrf2 regulates hepatosteatosis via autophagy.

Autophagosomes mature by fusing with lysosomes to form autophagolysosomes, where they are degraded. We evaluated the effect of Nrf2 on the fusion of lysosomes with autophagosomes, which was mediated by the lysosomal proteins LAMP1 and LAMP2[49], and found that Nrf2 deficiency-mediated LAMP1 inhibition impacted the fusion of autophagosomes and lysosomes in hepatocytes, resulting in the impairment of the autophagic degradation capacity of lysosomes and leading to hepatic lipid accumulation. To better understand the molecular mechanism by which Nrf2 deficiency inhibits LAMP1 expression, we performed ChIP-qPCR and dual luciferase assay, and the obtained findings confirmed that Nrf2 binds to LAMP1 promoter and regulates its transcriptional activity. A potential Nrf2-regulating site for the LAMP1 promoter was located at -1740 to -1726 bp upstream of the transcriptional start site.

As with other studies, even our study has some limitations: 1) we did not include Nrf2 hepatic-specific KO mice, mainly because they may still show low expression levels of Nrf2 in the liver, with the expression levels being potentially higher in other tissues (compensatory increase)[50], and 2) the potential mechanism for the interaction of Nrf2 with Atg7 remains to be determined.

Conclusions

To conclude, our data further reveal that Nrf2 deficiency promoted hepatosteatosis induced by HFD. Importantly, our study elucidated novel molecular mechanisms of Nrf2 regulating lipid metabolism in the liver through bioinformatics analysis and molecular biology technology, that is, Nrf2 can inhibit hepatic lipid accumulation by promoting autophagy through regulating LAMP1 transcriptional activity. Furthermore, we also provide genetic evidence for the interaction between Nrf2 and SREBP-1 (Fig. S2). Therefore, using an Nrf2 activator to promote the formation of autophagosomes and lysosomal acidification seems to be a promising strategy to treat MAFLD.

Abbreviations

ALT: Serum alanine transaminase

AST: aspartate transaminase

FAs: free fatty acids

HFD: high fat diet

MAFLD: metabolic associated fatty liver disease

MASH: metabolic associated steatohepatitis

MPHs: mouse primary hepatocytes

Nrf2: Nuclear factor erythroid 2-related factor 2

Nrf2 KO: Nrf2 knockout

NCD: chow diet

TG: triglycerides

TC: Serum total cholesterol

WT: C57BL/6J mice

Declarations

Ethics approval and consent to participate

This study was approved by Chongqing Medical University Animal Studies Committee and the methods were carried out in accordance with the approved guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

No potential conflicts of interest relevant to this article were reported.

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Author Contributions.

S.Q., Z.L., M.W., M.Y. and Q.W. researched and analyzed the data. C.C. and Z.M. contributed to the writing of the manuscript and helpful discussion. H.Z. provided research material and directed the project and contributed to the discussion. L. L. and G. Y. wrote and edited the manuscript. L. L. and G. Y. were the guarantors of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figures

Figure 1

Hepatic Nrf2 is up-regulated in patients with NAFLD and obese mice. (A, B) Nrf2 mRNA (A) and protein (B) expression in the liver of patients with NAFLD. (c, d) Nrf2 mRNA (C) and protein (D) expression in the liver of NCD- or HFD-fed WT mice. (E) IHC staining of liver sections from NCD- or HFD-fed C57BL/6J mice. (F, G) MPHs from WT mice were treated with BSA or FAs for 24h. Nrf2 mRNA (F) and protein (G) expression. NC, normal controls; NCD, normal chow diet; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease;

MPHs, mouse primary hepatocytes. The data are expressed as means $\pm$ SEM. western blot: n = 3-4/group; animal experiments: n = 5-6/group. ** p < 0.01 vs. NC, NCD, or BSA.

Figure 2

Nrf2 deficiency facilitates hepatic steatosis induced by HFD. 8-week-old WT and Nrf2 KO mice were fed an HFD or NCD for 12 weeks. (A) Schematic diagram of experiment design. (B) Body weight. (C) Representative livers at the macroscopic examination. (D) Liver weight. (E) Liver weight/body weight. (F-G) H&E(f) and Oil Red O (g) staining of liver sections. (H) Hepatic TG contents. (I) Serum TG levels. (J) Serum AST levels. (K) Serum ALT levels. (L) Serum CHO levels. HFD, high-fat diet; NCD, normal chow diet;

TG, triglyceride; AST, aspartate transaminase; ALT, serum alanine transaminase; CHO, serum cholesterol. Data are expressed as means $\pm$ SEM. western blot: n = 3/group; animal experiments: n = 5-6/group. * p < 0.05, ** p < 0.01 vs. HFD-fed WT.

Figure 3

Effects of Nrf2 deficiency on lipogenesis in the livers. (A) GSEA enrichment analysis indicating a relationship between Nrf2 expression and lipid homeostasis. (B) BP enrichment analysis from the GSE35124 dataset. (C) 8-week-old WT and Nrf2 KO mice were fed an HFD or NCD for 12 weeks. Immunoblot analysis of gene expression in the liver. (D-L) MPHs from WT and Nrf2 KO mice and HepG2 cells were incubated with BSA or FAs for 24h. Oil red O staining (D, E), TG contents (F, G), and Immunoblot analysis of gene expression (H, I). MPH, mouse primary hepatocyte; TG, triglyceride; FAs, fatty acid mixture; Data are expressed as means $\pm$ SEM. western blot: n = 3-4/group; animal experiments: n = 5-6/group. * p < 0.05, ** p < 0.01 vs. HFD-WT, FAs-WT or FAs-DMSO.

Figure 4

SREBP-1c is needed for the effect of Nrf2 on lipogenesis. (A) MPHs from WT and SREBP-1c KO mice were incubated with BSA or FAs for 24h and then treated with DMSO or ML385. (B) TG content. (C) Oil red O staining. (D) FAS and (E) SCD-1 mRNA expression. ML385, an Nrf2 inhibitor; Data are expressed as means $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. FAs- DMSO.

Figure 5

Nrf2 deficiency attenuates autophagy in the liver of mice fed an HFD. (A) GSEA enrichment analysis indicating the relationship between Nrf2 expression and lysosome function. (B-D) Male Nrf2 KO and WT mice were fed an HFD or NCD for 12 weeks. (B) LC3II, p62 and Atg protein expression in the livers. (C) IHC staining of liver sections for LC3 and p62. (D) LAMP1 and LAMP2 protein expression in the liver. Data are

expressed as means $\pm$ SEM. western blot: n = 3/group; animal experiments: n = 5-6/group. * p < 0.01, ** p < 0.01 vs. HFD-WT.

Figure 6

Nrf2 deficiency attenuates autophagy in vitro. (A, B) MPHs from Nrf2 KO and WT mice were treated with FAs or BSA for 24 h. (A) p62 protein expression and LC3II densitometry. (B) Atg and LAMP expression at protein levels. (C) HepG2 cells were incubated with BSA or FAs for 24h and then treated with DMSO or

ML385. LC3II densitometry, p62, Atg and LAMP protein expression; (D) HepG2 cells and MPHs were transfected with Ad-mRFP-GFP-LC3 and then exposed to BSA or FAs for 24h. Confocal images and quantification of LC3 reporter; All images were obtained using a 90× objective. MPH, mouse primary hepatocyte; FAs, fatty acid mixture. Data are expressed as means ± SEM from three independent experiments. * $p < 0.01$, ** $p < 0.01$ vs. FAs-WT or FAs-DMSO.

Figure 7

Nrf2 inhibits autophagy via the transcriptional regulation of LAMP1 promoter. (A) A schematic diagram for predicting the binding site of Nrf2 and LAMP1 promoter. (B) ChIP-qPCR analysis of the binding region of Nrf2 and the LAMP1 promoter. (C) Gel image and quantitative analysis of the ChIP DNA. (D) Luciferase reporter assay of LAMP1 transcriptional activity. Each bar represents the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. IgG or WT.

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