Hepatocyte-specific Nrf2 deficiency mitigates high-fat diet-induced hepatic steatosis: Involvement of reduced PPARγ expression

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A R T I C L E   I N F O

Keywords:
Nrf2
NAFLD
High-fat diet
Hepatocyte
PPARγ

A B S T R A C T

Non-alcoholic fatty liver disease (NAFLD) is an emerging global disease with increasing prevalence. However, the mechanism of NAFLD development is not fully understood. To elucidate the cell-specific role of nuclear factor erythroid-derived 2-like 2 (NRF2) in the pathogenesis of NAFLD, we utilized hepatocyte- and macrophage-specific Nrf2-knockout (Nrf2(L)-KO and Nrf2(M)-KO) mice to examine the progress of NAFLD induced by high-fat diet (HFD). Compared to Nrf2-LoxP littermates, Nrf2(L)-KO mice showed less liver enlargement, milder inflammation and less hepatic steatosis after HFD feeding. In contrast, Nrf2(M)-KO mice displayed no significant difference in HFD-induced hepatic steatosis from Nrf2-LoxP control mice. Mechanistic investigations revealed that Nrf2 deficiency in hepatocytes dampens the expression of peroxisome proliferator-activated receptor γ (PPARγ) and its downstream lipogenic genes in the liver and/or primary hepatocytes induced by HFD and palmitate exposure, respectively. While PPARγ agonists augmented PPARγ expression and its transcriptional activity in primary hepatocytes in a Nrf2-dependent manner, forced overexpression of PPARγ1 or γ2 distinctly reversed the decreased expression of their downstream genes fatty acid binding protein 4, lipoprotein lipase and/or fatty acid synthase caused by Nrf2 deficiency. We conclude that Nrf2-dependent expression of PPARγ in hepatocytes is a critical initiating process in the development of NAFLD, suggesting that inhibition of Nrf2 specifically in hepatocytes may be a valuable approach to prevent the disease.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as a metabolic disease related to obesity and type 2 diabetes mellitus, with a diagnostic hallmark of hepatic triglyceride (TG) accumulation above 5% without alcohol consumption [1]. NAFLD is currently a common liver disease with a growing prevalence worldwide. A recent meta-analysis reported that the global prevalence of NAFLD was up to 25% in 2016 [2]. NAFLD can be divided into simple steatosis and steatohepatitis (NASH) [3]. When wide-range inflammation and fibrosis occur, about 10–30% of the cases of simple steatosis develop into NASH [4]. Without effective intervention, 10–29% NASH might progress into cirrhosis in around 10 years [5]. The progression of cirrhosis is dramatic, and 27% of cases, can lead to hepatocellular carcinoma or death without liver transplantation [6]. Therefore, medical intervention of NAFLD should begin as early as possible after diagnosis.

NAFLD is mainly caused by disruption of the homeostasis of lipid metabolism, oxidative stress and subsequent inflammation. Nuclear factor erythroid-derived 2-like 2 (NFE2L2, also well known as NRF2) is a master transcription factor in the regulation of anti-oxidative and anti-inflammatory genes [7]. NRF2 is a member of CNC-bZIP family that binds to the antioxidant response element with small Maf proteins.
transgenic mice expressing Cre recombinase under the control of bumin mouse models established by crossing Nrf2 with lower liver weight and hepatic TG levels. However, this evidence from a lipopolysaccharide-stimulated anti-in mouse model [9] supports a role of NRF2 as an essential regulator of then fed with HFD to examine the e Nrf2 cytes or macrophages on the development of NAFLD. Compared with to trigger the transcription of downstream genes [8]. In addition, evidence from a lipopolysaccharide-stimulated anti-inflammatory genes, since NRF2-dependent transcription inhibits the activation of nuclear factor kappa-B (NF-kB) and expression of its downstream proinflammatory cytokines, such as inducible nitric oxide synthases, tumor necrosis factor α (TNFα), interleukin 1 beta (IL1β) and interleukin 6 (IL6) [9]. The molecular mechanism by which NRF2 suppresses inflammatory response is demonstrated to be disruption of proinflammatory cytokines using chromatin immunoprecipitation (ChIP)-seq and ChIP-qPCR analyses in macrophages [10]. In a high-fat diet (HFD)-induced NAFLD model, levels of oxidative stress and inflammation were both increased in the liver of Nrf2-KO mice that were susceptible to NAFLD [11,12]. Moreover, oxidative stress and inflammation appear to be in a vicious cycle, aggravating each other to induce disease progression [13,14].

Aspects of hepatic lipid metabolism associated with NAFLD development includes free fatty acid (FFA) uptake and transport, de novo synthesis, β-oxidation and exportation of TG out of hepatocytes. NRF2 is closely related to lipid homeostasis [7]. Utilizing a transgenic mouse model, many enzymes in these processes were identified and shown to be regulated by NRF2, such as cluster of differentiation 36 (CD36) [15]. Peroxisome proliferator-activated receptor γ (PPARγ) is a classic lipid metabolism regulator in adipocytes and hepatocytes, which is found as an initial factor in the development of NAFLD [16]. Our previous studies indicate that PPARγ is a direct downstream transcriptional target of NRF2 in adipocytes [17]. Hepatic steatosis is mainly attributed to hepatocytes [18], while Kupffer cells and stellate cells also contribute to the development of NAFLD [19,20]. Kupffer cells, specialized macrophages located in the liver, might be activated by abnormal lipid accumulation and injury in hepatocytes leading to the development of NASHI [21]. Increasing transcription of pro-inflammatory cytokines, such as TNFα, in Kupffer cells, might activate the stellate cells, leading to collagen synthesis and fibrosis and ultimately cirrhosis. While different types of cells in the liver play distinct roles in the pathogenesis of NAFLD, the initiator of this pathological process is abnormal lipid metabolism within hepatocytes.

In this study, we utilized hepatocyte-specific Nrf2-knockout (Nrf2-LKO) and macrophage-specific Nrf2-knockout (Nrf2(Mφ)-KO) mouse models established by crossing Nrf2-LoxP mice [22] with transgenic mice expressing Cre recombinase under the control of Albumin (Alb-Cre) and Lysozyme 2 (Lyz-Cre), respectively. The mice were then fed with HFD to examine the effect of Nrf2 deficiency in hepatocytes or macrophages on the development of NAFLD. Compared with Nrf2-LoxP littermate controls, Nrf2(LKO) mice had milder NAFLD, with lower liver weight and hepatic TG levels. However, this effect was not seen in Nrf2(Mφ)-KO mice. This mitigation of hepatic steatosis was accompanied by decreased PPARγ activity in Nrf2(LKO) mice. Additional mechanistic investigations in primary hepatocytes indicated that Nrf2-dependent expression of PPARγ plays a critical role in the initiation of NAFLD.

2. Materials and methods

2.1. Animals

Nrf2(LKO) mice and Nrf2(Mφ)-KO mice on a C57BL/6 background were generated by breeding Nrf2(LKO) mice with Albumin-Cre mice (Nanjing Biomedical Research Institute of Nanjing University, J003574) and Lysozyme2-Cre mice (Nanjing Biomedical Research Institute of Nanjing University, J004781), respectively. All breeders and littermates were raised at the animal facility of China Medical University. All animal experiments performed were approved by the Institutional Animal Care and Use Committee of China Medical University (Shenyang, China). Mice were housed at 23 ± 1 °C and kept on 12-h light/dark cycles (lights on: 06:00–18:00). Distilled water and standard mouse chow diet (CD, Shukebeita Specific Pathogen Free Mouse Maintenance Diet, Kietong Organism, Jiangsu, China) were provided ad libitum. Tail snips were collected to prepare genomic DNA for genotyping, as described previously [22].

Both female and male 12–16 weeks old Nrf2(LKO)-mice and littermate control Nrf2-LoxP mice were randomly divided into 2 groups, respectively. The mice in Nrf2-LoxP, CD and Nrf2(LKO), CD groups were provided with standard CD. In contrast, the mice in Nrf2-LoxP, HFD and Nrf2(LKO), HFD groups were fed with a HFD (60% kcal fat; Research diets #D12492) for 12 weeks. Distilled water was provided ad libitum to all mice. Food and water consumption, body weight and blood glucose levels were monitored regularly. At necropsy, the liver was weighed and a portion was excised and fixed in 4% paraformaldehyde buffer for histopathology. Blood and various tissue samples were collected and frozen at −80 °C.

2.2. Intraperitoneal glucose tolerance test (IPGTT)

The mice fed with HFD were given an intraperitoneal injection of 1.0 g/kg BW of D(+)-glucose (G8769; Sigma, St. Louis, MO) following overnight fasting, and blood glucose levels were measured at 0, 15, 30, 60 and 120 min after the glucose injection using the FreeStyle Blood Glucose Monitoring System (TheraSense, Alameda, CA). Blood samples were collected from the tail bleeds and analyzed as described previously [22].
2.3. Measurement of body composition

The fat mass of mice was measured by a Minispec LF-50 NMR body composition analyzer with the Minispec NF software (Bruker BioSpin GmbH, Rheinstetten, Germany) according to the manufacture’s protocol [23].

2.4. Analysis of TG, glycerol and free fatty acids (FFA)

Heparin-treated fresh blood samples were centrifuged at 5,000 g for 5 min at 4 °C. The liver samples or the primary hepatocytes were homogenized in cold PBS (or chloroform:methanol = 2:1) and then centrifuged at 12,000 g for 5 min at 4 °C. The resultant supernatants were used for measurements. The levels of glycerol and total TG in the plasma samples and liver supernatants were assessed using specific kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) adhering by the manufacturer’s instructions. FFA levels were measured using a standard protocol of a mouse enzyme linked immunosorbent assay (ELISA) kit (Jiangsu Meimian Industrial Co., Ltd, Jiangsu, China).

2.5. Measurement of malondialdehyde (MDA) levels

The liver tissues were homogenized in the lysis buffer (#P0013, Beyotime Institute of Biotechnology, Shanghai, China). Total protein content was determined by using the Enhanced BCA Protein Assay Kit (#P0009, Beyotime). MDA levels were measured with Lipid Peroxidation MDA assay kit according to the instructions (#S0131, Beyotime).

2.6. Liver histological and immunohistochemical analysis

Liver samples were fixed in 4% paraformaldehyde buffer overnight after harvesting and then transferred to 70% ethanol. After dehydrating with graded ethanol solutions, tissues were then embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin-eosin stain (H&E). For Oil Red O staining, 20 μm thickness frozen section was used. The stained sections were assessed visually by light microscopy.

For immunohistochemistry, the macrophage marker F4/80 (sc-377009, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) were assessed following IHC-DAB kit instructions (Beijing Zhongshan Jinqiao Biological Technology Co., Beijing, China) as described previously [24].

2.7. Primary hepatocytes isolation and treatments

Primary hepatocytes were isolated from Nrf2-LoxP and Nrf2(L)-KO mice fed with CD. A modified collagenase method for hepatocyte isolation described previously [25] was utilized. In brief, mice were first anaesthetized with pentobarbital sodium (50 mg/kg BW). Then the liver was perfused with Hank’s balanced salt solution (37 °C) via the vena cava, followed by a collagenase buffer (1 mg/mL; C-5138, Sigma) for cell dissociation. Digested livers were centrifuged at 30 g for 3 min to concentrate pure hepatocytes at the bottom of tubes. Isolated primary hepatocytes were cultured in M199 medium with 100 units/ml penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. After culture for 6 h, primary hepatocytes were treated with rosiglitazone (ROSi, Beijing Solarbio Science & Technology Co., Ltd, Beijing, China; 0, 1, 10 μM) and pioglitazone (PIOG, Beijing Solarbio; 0, 0.25, 0.5 μM) for 24 h, respectively.

Sodium palmitate (P9767, Sigma) was dissolved to 0.15 M in 0.1 mM NaOH solution via heating at 90 °C and oscillating for 5 min. 10% BSA (abs49001012, Absin, Shanghai, China) solution was immediately added to make 15 mM and filtered with 0.22 μm filter as stock (pH = 7.65). Before application to isolated primary hepatocytes, stock solution was incubated in a water bath at 37 °C for conjugation, and then diluted to 0.5 mM into M199 medium.

2.8. Measurement of fatty acid uptake

The Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent, Billerica, MA, USA) was used to measure OCR and ECAR as previously described [27]. Isolated primary hepatocytes from Nrf2(L)-KO and Nrf2-LoxP mice were seeded in XF24-well microplates at 5.0 × 10⁴ cells per well. For 6 h culture (without FBS), the medium was replaced with 10 μg/L BODIPY in 200 μL PBS. The fluorescence values were measured immediately utilizing fluorescence microplate reader (Molecular Devices FlexStation 3) with kinetic mode for 45 min (once every 3 min, totally 16 reads). The excitation and emission wavelengths of BODIPY were set to 493 nm and 520 nm, respectively.

2.9. Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in primary hepatocytes

Liver steatosis analyzed based on NAS.

| NAS Steatosis levels | CD | HFD |
|---------------------|----|-----|
| Nrf2-LoxP | Nrf2(L)-KO | Nrf2-LoxP | Nrf2(L)-KO |
| 0: < 5% | 10 | 11 | 0 | 0 |
| 1: 5%–33% | 0 | 0 | 2 | 5 |
| 2: 33%–67% | 0 | 0 | 4 | 2 |
| 3: > 67% | 0 | 0 | 2 | 0 |

CD: chow diet; HFD: high-fat diet; NAS: NAFLD Activity Score.

Note: 16 wks old Nrf2-LoxP and Nrf2(L)-KO female mice were fed with CD and HFD for 12 wks and the steatosis of livers were evaluated based on H&E staining. Based on NAS, the primary histologic features were classified into four grades of steatosis severity (% cells involved in lesion; evaluation of parenchymal involvement by steatosis) [32]. According to the Kruskal-Wallis test, p = 0.058 in HFD fed Nrf2(L)-KO vs Nrf2-LoxP mice.

2.10. Overexpression of PPARγ in hepatocytes

Transient transfection was performed in primary hepatocytes isolated from Nrf2(L)-KO and Nrf2-LoxP mice fed with CD with Lipofectamine 3000 (Life Technologies, Grand Island, NY) following standard procedures [25]. Briefly, 2.5 μg plasmids in 7.5 μL Lipofectamine 3000 were administrated to primary hepatocytes of both genotypes for 48 h. The PPARγ plasmid was purchased from Addgene (plasmid #8886; Cambridge, MA) [28] and the PPARγ2 with HA tag was gifted from Dr. Wen Xie (University of Pittsburgh) [29].

2.11. Western blotting

Liver tissues and harvested cells were prepared and lysed according to standard protocols as previously described [22,30]. Antibodies for PPARγ (#2435; 1:1000; Cell Signaling Technology (CST), Danvers, MA), pNF-κB (#3033; 1:1000; CST), NF-κB (#8242; 1:1000; CST),
PPARα (ab8934; 1:1000; Abcam, Cambridge, United Kingdom), NRF2 (sc-13032; 1:800; Santa Cruz Biotechnology, CA), α-TUBULIN (sc-5286; 1:1000, Santa Cruz) and β-ACTIN (sc-47778; 1:1000; Santa Cruz) were used. Blotting membranes were incubated with the primary antibody at 4 °C overnight and the secondary IgG-horse radish peroxidase (#31460; 1:10000; Thermo Scientific) at room temperature for 1 h. The resulting bands were visualized using Tanon 5500 (Tanon, Shanghai, China) as previously described [30]. Quantification of the results was accomplished using ImageJ software (National Institute of Mental Health, USA).

2.12. RT-qPCR

Liver tissues were lysed using TissueLyser II (#85300, Qiagen, Germany). Total RNA was isolated from liver lysates and primary hepatocytes with RNAiso Plus kits (Code No. 9109, Takara, Dalian, China). cDNA was generated using Prime Script RT reagent Kits (Takara, Dalian, China). Real-time PCR reactions were performed using the SYBR Premix EX Taq Kit (Takara, Dalian, China) and the QuantiStudio 6 Flex real-time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) [22,24]. cDNA was amplified using the following PCR conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed using the 2−ΔΔCT method as previously described [31]. Primers for Pparg1/2, fatty acid binding protein 4 (Fabp4), Cd36, sterol regulatory element-binding transcription factor 1 (Srebf1), MLX interacting protein like (Mlxipl), fatty acid synthase (Fat), stearoyl-CoA desaturase-1 (Scd1), Actin and other genes are listed in supplementary Table S1. Primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (China). All mRNA levels were presented relative to Actb of Nrf2−LoxP mice fed with CD or control untreated hepatocytes.

2.13. Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 6 software (Graphpad Software Inc., San Diego, CA) with p < 0.05 considered as significant. Statistical differences were determined by an unpaired Student’s t-test or by two-way analysis of variance followed by a Dunnett’s multiple comparison test. Analysis for Table 1 was performed using Statistical Product and Services software (v.16.0, SPSS Institute Cary, Chicago, IL, USA) with a Kruskal-Wallis test for comparing all groups and a Mann-Whitney test for the comparison of two specific groups.

3. Results

3.1. Nrf2(L)-KO mice fed with HFD show smaller livers, milder hepatic steatosis and less hepatic TG accumulation

To confirm the knockout efficiency of Nrf2(L)-KO mice, an
immunoblotting assay against NRF2 was performed using the whole tissue lysates of liver. As shown in Fig. 1A and B, the protein levels of NRF2 were dramatically decreased in the liver of Nrf2(L)-KO mice relative to Nrf2-LoxP littermate controls. To determine the potential effect of NRF2 ablation in hepatocytes on general energy metabolism, the body weight, food intake, water consumption, fat mass and blood glucose levels were monitored over 12 weeks for all groups. As shown in Fig. 1C, caloric intake was calculated based on food consumption and the energy content of the diets (CD, 3.616 kcal/g; HFD, 5.243 kcal/g). While the energy intake of HFD-fed mice were significantly higher than those of CD groups, there were no significance between genotypes, either under CD or HFD. In addition, the water consumption also showed minimal fluctuation among the 4 groups (Fig. 1D). Furthermore, Nrf2(L)-KO mice fed with either CD or HFD showed no significant differences in body weight gain (Fig. 1E), relative fat mass measured by a body composition analyzer (Fig. 1F) and fasting blood glucose levels (Fig. 1G) from the corresponding littermate controls, whereas the mice responded well to HFD exposure, showing elevated body weight gain, relative fat mass and blood glucose levels compared to CD controls. In agreement with the measurements above, IPGTT performed in the HFD groups following HFD exposure also displayed no significant difference between the genotypes (Fig. 1H and I). The same tendency was also seen in the measurements of male Nrf2(L)-KO mice (Supplementary Fig. S1).

To further ascertain the effect of NRF2 deletion on body composition, the organ mass indexes were determined in Nrf2(L)-KO mice and their Nrf2-LoxP controls following HFD exposure. Because the heart weight showed no significant difference between the genotypes, either treated with CD or HFD (Fig. 2A), we used the heart weight instead of body weight to calculate relative organ weight in order to eliminate the possible bias of altered amounts of fat tissues (Fig. 2B). Compared to CD, consumption of HFD by control mice resulted in enlargement of the liver. Nrf2(L)-KO mice fed with HFD showed significantly less increase in liver size than control mice (Fig. 2B and S1F). In contrast, there were no significant differences in the mass of adipose tissues between the genotypes under either CD- or HFD-fed conditions. In addition, there were no significant differences in the levels of TG, glycerol and FFA in plasma among various situations (Fig. 2C–E). In contrast, hepatic TG, but not glycerol and FFA, in Nrf2-LoxP mice exposed to HFD were significantly higher than those in control mice treated with CD (Fig. 2F–H). Interestingly, hepatic TG in the Nrf2(L)-KO mice fed with HFD were significantly lower than those in the Nrf2-LoxP mice on the same diet (Fig. 2F). In addition, the livers of mice fed with HFD generally had more fat deposition evaluated by histological analysis than those fed with CD, whereas Nrf2(L)-KO mice fed with HFD had less hepatic lipid deposition than their Nrf2-LoxP littermates with the same diet (Fig. 2I and S2). A severity analysis of liver steatosis calculated by a NAFLD Activity Score as described previously [32], also displayed a
relative attenuated steatosis in HFD-fed Nrf2(L)-KO mice compared to Nrf2-LoxP controls (Table 1). Taken together, the analyses above clearly elucidated that Nrf2(L)-KO mice are resistant to HFD-induced hepatic steatosis compared to Nrf2-LoxP control mice.

3.2. Nrf2(L)-KO mice have attenuated expression of PPARγ2 induced by HFD exposure in the liver

To understand the mechanism that Nrf2(L)-KO mice become resistant to HFD-induced hepatic steatosis, the expression of mRNA related to oxidative stress, glucose and lipid metabolism, inflammation and fibrosis was measured by RT-qPCR (Fig. 3A and B). Consistent with the deficiency of Nrf2 in hepatocytes, the mRNA levels of Nrf2 and its downstream genes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutamate-cysteine ligase catalytic subunit (Gclc) and hemeoxygenase 1 (Hoe1), were all decreased in the liver of Nrf2(L)-KO mice compared to Nrf2-LoxP under CD or HFD exposure, while HFD had no significant effect on these mRNA expression. The expression of the rate-limiting enzyme of β-oxidation, carnitine palmitoyl transferase (Cpt), was reduced to nearly 50% by HFD exposure in both Nrf2-LoxP and Nrf2(L)-KO genotypes, which is consistent with TG accumulation in hepatocytes following HFD exposure (Fig. 3A). In contrast, the mRNA levels of pro-inflammatory cytokines, including Ccl2 and Tnf, were significantly induced by HFD exposure in both Nrf2(L)-KO and control mice, but there were no differences between the genotypes either under CD or HFD (Fig. 3A). Hepatic mRNA level of a key lipid synthesis gene, Srebf1, was increased in HFD-fed Nrf2(L)-KO mice compared to control mice with the same diet, which is not consistent with the reduced liver steatosis seen in HFD-fed Nrf2(L)-KO mice (Fig. 3B). This finding suggests that other mechanism(s) might play more dominant role in the regulation of lipid metabolism in Nrf2(L)-KO hepatocytes. Importantly, the levels of Pparγ2 mRNA were found to be induced by HFD exposure in both genotypes, whereas the expression of Pparγ2 showed a trend to be lower in Nrf2(L)-KO mice compared to control mice (Fig. 3B). No significant differences between the Nrf2(L)-KO and their littermate controls were noted for any other measured genes (Fig. 3A and B). In contrast, the mRNA expression, the protein levels of Nrf2 in the liver showed a dramatic reduction in Nrf2(L)-KO mice compared to controls (Fig. 3C and D). In contrast, the MDA levels in the liver showed no significant difference between the two genotype mice with CD or HFD (Fig. 3E). Consistent with the mRNA expression of Pparγ2, the protein levels of PPARγ2 in the liver of Nrf2(L)-KO mice fed with HFD were also lower than those in Nrf2-LoxP group (Fig. 3F–H). In addition, that the levels of phosphorylated NF-κB p65, but not total NF-κB p65, were substantially induced in the liver of Nrf2-LoxP mice by HFD exposure, whereas Nrf2(L)-KO mice showed a lower expression compared to control mice with the same diet (Fig. 3F). Furthermore, immunohistochemistry analysis against F4/80, a macrophage marker, showed that HFD exposure induced macrophage infiltration in the livers of Nrf2-LoxP control mice (Fig. 3I). Compared to control mice, Nrf2(L)-KO mice have less hepatic macrophage infiltration following HFD exposure (Fig. 3I).

3.3. Nrf2(M4)-KO mice display no significant difference in hepatic steatosis from Nrf2-LoxP control mice

To further clarify that Nrf2 in hepatocytes play a critical role in the development of hepatic steatosis, a parallel investigation was conducted in Nrf2(M4)-KO mice and their Nrf2-LoxP controls. As shown in Fig. 4, there were no significant differences in calorie intake, water consumption, body weight, relative fat mass, blood glucose level, glucose tolerance test, major organ mass index, and hepatic lipid accumulation between the genotypes under either CD or HFD exposure conditions, whereas HFD exposure significantly elevated most of those parameters in Nrf2(M4)-KO and Nrf2-LoxP control mice (Fig. 4A–K).

To further validate the phenotype of Nrf2(M4)-KO mice, we measured the mRNA levels of oxidative stress and inflammation-related genes in the liver. As shown in Fig. 4L, the mRNA expression of Ccl2 and Tnf in the liver was significantly elevated by HFD exposure in both genotypes of mice. However, there were no significant difference in the mRNA expression between the genotypes either under CD or HFD exposure. Other mRNAs measured showed no significance among the four groups.

3.4. PPARγ agonists increase the expression of PPARγ and its downstream target genes in primary hepatocytes in a Nrf2-dependent manner

To investigate the role of PPARγ in lipid deposition in Nrf2-deficient hepatocytes, the PPARγ agonists, ROSI and PIOG, were used in the primary hepatocytes isolated from Nrf2-LoxP and Nrf2(L)-KO mice. As shown in Fig. 5, the mRNA and protein expression of PPARγ and PPARγ2 were significantly induced by ROSI (Fig. 5A and B) or PIOG (Fig. 5C and D) in Nrf2-LoxP hepatocytes. Nrf2(L)-KO hepatocytes showed dramatically attenuated mRNA and protein expression of PPARγ and PPARγ2 under vehicle or agonist-challenged conditions. In the addition, the mRNA expression of Fabp4 and Cd36, which are known PPARγ target genes, Scd1 and Srebf1 displayed a similar pattern as PPARγ (Fig. 5E and F), suggesting that Nrf2 is critical in PPARγ activation in hepatocytes.

3.5. Nrf2 deficiency down-regulates PPARγ activation and lipogenesis induced by palmitate treatment in primary hepatocytes

Primary hepatocytes isolated from Nrf2(L)-KO and Nrf2-LoxP control mice were treated with palmitate (0.5 mM) to simulate PPARγ and subsequent lipogenesis. The mRNA levels of Pparγ1 and Pparγ2 increased and peaked at 2 h after treatment (Fig. 6A and B), and decreased thereafter (6, 12 and 24 h). Compared to Nrf2-LoxP cells, Nrf2(L)-KO hepatocytes were less responsive in their Pparγ1 and Pparγ2 mRNA induction. In agreement with the mRNA expression, the protein levels of PPARγ1 and PPARγ2 also displayed a trend of increase in both genotypes, while the Nrf2 deletion weakened the tendency (Fig. 6C–E). In addition, the mRNA expression of Fabp4, Scd1 and Fasn, the major downstream lipogenic genes of PPARγ, also showed significant increases in response to palmitate treatment in Nrf2-LoxP cells, whereas Nrf2(L)-KO hepatocytes had reduced induction in their expression (Fig. 6F–H). Following a 24-hrs palmitate treatment, the TG levels in Nrf2(L)-KO hepatocytes were much lower than those in Nrf2-LoxP cells (Fig. 6I and Fig. 5A).

To evaluate the effect of Nrf2 deficiency on fatty acid uptake in hepatocytes, a BODIPY uptake assay was performed. As shown in Fig. 6J, the rate of BODIPY uptake in Nrf2(L)-KO hepatocytes was slower than control cells. In contrast, the mitochondrial function in hepatocytes, as measured by OCR and ECAR, showed no significant difference between the two types of cells (Fig. 6K and L).
Fig. 4. Measurements in Nrf2(Mϕ)-KO mice and Nrf2-LoxP littermates. (A–C) Calorie intake (A), water consumption (B) and body weight (C) during the HFD exposure period. Age-matched Nrf2-LoxP and Nrf2(Mϕ)-KO mice were fed with CD or HFD diet for 12 wks from 16 wks of age. *p < 0.05 vs. CD of the same genotype. (D) Fat mass as percentages of body weight measured by a Body Composition Analyzer. (E) Fasting blood glucose levels. n = 5 for Nrf2-LoxP, CD; n = 9 for Nrf2(Mϕ)-KO, CD; n = 9 for Nrf2-LoxP, HFD; n = 13 for Nrf2(Mϕ)-KO, HFD. (F) Intraperitoneal glucose tolerance test following HFD exposure. Nrf2(L)-KO and control mice fed with HFD 12 wks were challenged with 1.0 mg of glucose per gram of BW. n = 9–13. (G) Areas under the curves of (F). (H–I) Heart mass (H) and organ mass index measured as a fold of heart (I). n = 5–13. (J) Levels of hepatic TG. n = 5 for Nrf2-LoxP, CD; n = 3 for Nrf2(Mϕ)-KO, CD; n = 6 for Nrf2-LoxP, HFD; n = 5 for Nrf2(Mϕ)-KO, HFD. (K) Representative histological images of the H&E staining in the liver. Magnification = 100 ×; bar scale = 300 μm. (L) Hepatic mRNA levels in Nrf2(Mϕ)-KO and Nrf2-LoxP control mice.
3.6. Overexpression of PPARγ1 or γ2 distinctively reverses the reduced expression of lipogenic genes caused by Nrf2 deficiency in primary hepatocytes

To validate the involvement of PPARγ1 or 2 in the alteration of lipogenic genes in Nrf2(L)-KO hepatocytes, we overexpressed Pparg1 and Pparg2 in primary hepatocytes from Nrf2(L)-KO and Nrf2-LoxP mice. As shown in Fig. 7A and B, both mRNA and protein levels were measured to confirm the efficacy of overexpression. Accordingly, the direct downstream gene of PPARγ, Fabp4, increased in both Nrf2-deleted and control hepatocytes to the same extent (Fig. 7C and D), which indicate that PPARγ1 or PPARγ2 overexpression reverses NRF2-dependent reduction of PPARγ activity. However, the reversal was not observed in the mRNA levels of Cd36, a direct target gene of NRF2 [33,34], suggesting that the relationship between CD36 and NRF2 was more prominent than CD36 and PPARγ in the process of hepatic steatosis. Consistent with this notion, the mRNA levels of Lpl and Scd1 and Fasn were also reversed by overexpression of Pparg1 (Fig. 7C) and Pparg2 (Fig. 7D), respectively. While other PPARγ target genes reported in adipose tissues were also induced by the overexpression, their expression were not fully reversed in Nrf2(L)-KO cells, compared to Nrf2-LoxP hepatocytes (Fig. 7C and D).

4. Discussion

Previous studies utilizing global Nrf2-KO or constitutive NRF2-activated mice showed that NRF2 plays a critical role in the development of NAFLD, in which NRF2 is involved in the regulation of lipid metabolism, inflammation and antioxidant response [7]. However, the conclusions from those models on whether, how and where NRF2 participates in the development of NAFLD are not quite clear, which might be partially due to the modifications of Nrf2 gene in those models.
occurred throughout the body, but not any cell type-specific. Here, we used hepatocyte- and macrophage-specific Nrf2-knockout mouse models to induce NAFLD with a prolonged HFD exposure. Nrf2 deletion in hepatocytes, but not in macrophages, resulted in attenuated hepatic steatosis. Mechanistic investigations indicate that the attenuation from HFD-induced NAFLD in Nrf2 (L)-KO mice might be attributable to the decreased expression of PPARγ, PPARγ2 in particular. These findings demonstrate that Nrf2-dependent expression of PPARγ plays a critical role in hepatocytes for the initiation of NAFLD induced by HFD exposure.

Consistent with our previous findings in adipocytes [17], the results in primary hepatocytes confirmed that PPARγ2 is down-regulated with Nrf2 deletion, in particular under the PPARγ agonist-challenged conditions. In addition, PPARγ1 is also expressed in the liver and exhibits the same tendency as PPARγ2 in Nrf2 (L)-KO hepatocytes and liver tissues. In a NAFLD model using hepatocyte-specific Pparg-knockout mice, a set of enzymes related to de novo lipogenesis were down-regulated, while PPARγ activation by an agonist might reverse the phenotype [35], indicating that PPARγ is a crucial factor for the initiation of NAFLD. The results in the present study indicate that PPARγ activation induced by PPARγ agonists (ROSI and PIOG) or palmitate treatment is dependent, at least in part, on the presence of Nrf2 in hepatocytes. Forced expression of either PPARγ1 or PPARγ2 was able to restore the alterations in the expression of lipogenic genes, such as Fabp4 and Lpl, in Nrf2 (L)-KO hepatocytes. Therefore, Nrf2-mediated activation of PPARγ and subsequent de novo lipogenesis are crucial for hepatic lipid accumulation. Since C321 is a downstream target gene of Nrf2, the mechanism on Nrf2 (L)-KO hepatocytes had decreased fatty acid uptake rate in response to FFA challenge might be a result of lowered expression of CD36 in the cells. These findings indicate that PPARγ is one of the major factors mediating Nrf2-regulated lipogenesis in hepatocytes. In addition, Nrf2 might also play a distinct role in hepatocytes to regulate lipid homeostasis in a PPARγ-independent way.

SREBP1 is a master regulator of de novo lipogenesis [36]. In the current study, Srebf1 was found to be the only gene elevated in HFD-fed Nrf2 (L)-KO mice compared to control group with the same diet. This is consistent with the results of many other studies using Nrf2 transgenic mouse models [11,37,38]. However, our Nrf2 (L)-KO mice fed with HFD showed lower liver TG accumulation, which highlights the important role of PPARγ in lipogenesis in hepatocytes. Keap1-knockdown mice, which makes Nrf2 constitutively active, fed with HFD for 24 weeks exhibited body weight gain, increased inflammation and lipogenic gene expression [39], indicating that elevated Nrf2 aggravates the NAFLD process. To some extent, these results were in accordance with our present study showing that Nrf2 appears to play a pathogenic role in the initiation of NAFLD. Other studies utilizing genetic Nrf2 models also
drew similar conclusions [15, 40, 41].

Mice fed with HFD developed hepatic inflammation in both Nrf2-LoxP and Nrf2(L)-KO genotypes. The inflammation in Nrf2(L)-KO mice fed with HFD was generally lower than the Nrf2-LoxP HFD group, as indicated by the subdued phosphorylation of NF-κB, the mRNA levels of proinflammatory cytokines Ccl2 and Tnf and reduced macrophage infiltration. Although NRF2 can repress inflammation [10], we believe that the difference of inflammation between genotypes fed with HFD is a consequence of reduced hepatic steatosis in Nrf2(L)-KO group in this study. Consistent with this conclusion, there was no significant difference of NAFLD determination in the Nrf2(Mϕ)-KO mouse model, which indicates that Nrf2 deficiency in macrophage is not the key in the initiation of NAFLD.

Previous studies using mice with global changes in Nrf2 expression displayed unexpected phenotypes with inconsistency in body and liver weights, as well as expression of lipid metabolism-related genes [42]. This may be due to the fact that Nrf2 expression in different cell types or organs is well controlled and varies due to diverse status or diseases. In vivo, there may be compensation for the loss of global Nrf2 balance by subtle changes in expression that alter phenotype and conceal the effect of NRF2 on a specific disease [43]. In agreement with this explanation, we could not find significant increases in hepatic MDA levels, NRF2 protein levels, the mRNA expression of Nrf2 and the target genes after HFD feeding, which is consistent with the findings reported by Zhang et al. [44]. A more recent study used adipocyte- and hepatocyte-specific Nrf2 deletion mouse models to explore the role of NRF2 in obesity [45]. After 170 days of HFD feeding, the mice with hepatocyte Nrf2 deletion showed lower plasma insulin levels and improved insulin sensitivity.

Fig. 7. The reduced expression of PPARγ-related genes in Nrf2(L)-KO hepatocytes is partially reversed by PPARγ overexpression. Hepatocytes isolated from Nrf2-LoxP and Nrf2(L)-KO mice were transfected with Pparg1 and Pparg2 plasmids. (A) mRNA levels of Pparg1 and Pparg2 in the cells. *p < 0.05 vs. Veh of the same genotype; #p < 0.05 vs. Nrf2-LoxP with the same treatment. (B) Representative images of immunoblots of PPARγ. (C) mRNA expression of PPARγ downstream gene Fabp4 and other lipid metabolism-related genes.
without changing hepatic TG accumulation [45], which is not in line with our current findings in Nrf2(-L)-KO mice with 12 weeks of HFD challenge. It suggests that Nrf2 in hepatocytes possibly plays different roles in the initiation or progression of various NAFLD stages in different HFD exposure situations.

In conclusion, Nrf2(-L)-KO mice showed many signs of diminished NAFLD after HFD feeding, including lower accumulation of TG in the liver and minimal liver weight gain. A key aspect of this appears to be the ability of Nrf2 to regulate PPARγ expression and influence lipid metabolism in hepatocytes (Fig. 8). Thus, Nrf2 in hepatocytes is a critical factor in the initiation of NAFLD and a potential intervention molecular target. Further study is needed to elucidate the specific stages of NAFLD with Nrf2 modulation as an effective prevention or therapeutic strategy.

Author contributions

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Read of pathology picture: Linlin Wang, Rui Zhao, Lu Li. Interpreted the data and wrote the manuscript: Lu Li, Jingqi Fu, Jingbo Pi.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China 81830099 (J.P.), 81573106 (J.P.), 81573187 (Y.X.) and 81402635 (J.F.), Shenyang Municipal Bureau of Science and Technology Support Program for Young Innovation Scholar (RC180207, J.F.), Liaoning Province Natural Science Foundation (20180530011, J.F.), the Startup Funding of China Medical University (J.P.), Liaoning Pandeng Scholar Program (J.P.), China Medical University Training Program for National Natural Science Fund for Excellent Young Scholars (YQ20170001, J.F.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101412.

Transparency document

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