Deletion of Nrf2 leads to hepatic insulin resistance via the activation of NF-κB in mice fed a high-fat diet

ZHENXIONG LIU1*, WEJJIA DOU1*, ZHEN NI2*, QINSHENG WEN1, RONG ZHANG3, MING QIN1, XUXIA WANG1, HUA TANG1, YING CAO1, JINGJIE WANG1 and SHUGUANG ZHAO1

1Department of Gastroenterology, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710038; 2Department of Digestion, General Hospital of Chengdu Military Command, Chengdu, Sichuan 610000; 3Department of Gastroenterology, Xianyang Hospital of Yan'an University, Xianyang, Shaanxi 712000, P.R. China

Received June 11, 2015; Accepted May 23, 2016

DOI: 10.3892/mmr.2016.5393

Abstract. Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. Insulin resistance (IR) is important in the development and progression of NAFLD. Nuclear erythroid 2-related factor 2 (Nrf2) has previously been reported to be a novel regulator in NAFLD. The present study determined that Nrf2 knockdown accelerated the onset of obesity and non-alcoholic steatohepatitis (NASH), via the induction of hepatic IR in mice fed a high-fat diet (HFD), which was confirmed by an increase in total and hepatic weight in Nrf2-null-HFD mice, in addition to marked structural disorder in liver tissues from the Nrf2-null-HFD group analyzed by histopathological examination. Subsequently, it was demonstrated that hepatic IR in Nrf2-null-HFD mice was influenced by oxidative stress; this was confirmed by an increase in malondialdehyde levels and a decrease in glutathione levels. In addition, it was determined that the induction of hepatic IR by Nrf2 knockdown in HFD-treated mice was regulated by activation of the nuclear factor-κB (NF-κB) signaling pathway, as detected by an increase in the expression levels of nuclear NF-κB, and its downstream effectors interleukin-6 and tumor necrosis factor-α. The present study provides insight into the function of Nrf2 in NAFLD, indicating that Nrf2 deletion may lead to hepatic IR by activation of NF-κB, which is often associated with oxidative stress. Therefore, activation of Nrf2 may limit disease progression and act as a therapeutic approach for the treatment of NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD), which includes a broad spectrum of liver pathologies ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), can lead to liver inflammation, cirrhosis and hepatocellular carcinoma (1). NAFLD is one of the most common chronic liver diseases worldwide, with an estimated prevalence of 20-30% in Western countries, reaching 75-100% in obese individuals (2). In China, the incidence of NAFLD has also increased rapidly in recent years. However, the exact mechanism underlying the progression of NAFLD remains unclear and an effective treatment has not yet been discovered.

The ‘two-hit’ mechanism is a classical theory used to explain the pathogenesis of NAFLD (3). The first ‘hit’ leads to the deposition of triglycerides and steatosis, and sensitizes the liver to the second ‘hit’, which involves the release of inflammatory cytokines and oxidative stress. Eventually, these factors lead to the development of NASH and cirrhosis. However, there is increasing evidence that NAFLD is the hepatic manifestation of the metabolic syndrome (4). Hepatic insulin resistance (IR) is the key pathophysiological hallmark of the metabolic syndrome, which is important for the progression of NAFLD (5). Insulin signaling pathways, mediated by impaired tyrosine phosphorylation of insulin receptor substrate (IRS) (6), have previously been reported to be important for the development of IR. The levels of tyrosine-phosphorylated IRS are regulated by serine-threonine kinase and are negatively correlated with its serine-phosphorylated form. In addition, inflammatory signals, such as IkappaB kinase (IKK)/nuclear factor-κB (NF-κB) and c-Jun N-terminal protein kinase 1, or proinflammatory cytokines, including...
interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α), are involved in IR via the phosphorylation of IRS1 (7,8). A previous study indicated that oxidative stress may also induce IR (9). The interaction between oxidative stress and IR may accelerate cellular injury, inflammation and even lead to hepatic fibrosis (10). Furthermore, continual increase of reactive oxygen species (ROS) levels may activate serine-threonine kinase cascades, leading to reductions in tyrosine phosphorylated IRS, consequently resulting in IR (11).

Nuclear erythroid 2-related factor 2 (Nrf2) regulates the expression of various antioxidant genes and detoxification enzymes against oxidative and electrophilic stress (12), and has been regarded as a novel therapeutic target in liver diseases, including NAFLD (13). Under homeostatic conditions, Nrf2 is sequestered in the cytosol by binding to Kelch-like ECH-associated protein (Keap1) and forming a Nrf2-Keap1 complex. However, when exposed to oxidative or electrophilic stress, Nrf2 dissociates with Keap1, translocates into the nucleus, and activates numerous downstream genes (14). It has been demonstrated that Nrf2 deletion may aggravate inflammation and promote progression of NAFLD to NASH by inducing the nuclear translocation of NF-κB p65 protein and the expression of IL-1β, TNF-α and cyclooxygenase-2 in transgenic mice fed a methionine-choline deficient (MCD) diet (15). However, to the best of our knowledge, the effects of Nrf2 on hepatic IR in NAFLD have yet to be elucidated.

The present study investigated the biological function and underlying molecular mechanisms of Nrf2 in the development and progression of nutritional steatohepatitis. Initially, it was demonstrated that Nrf2 knockdown led to obesity and steatohepatitis induced by a high-fat diet (HFD). Nrf2 deletion may influence the initiation of NASH by promoting hepatic IR. Finally, it was confirmed that activation of NF-κB and its downstream effectors IL-6 and TNF-α, via Nrf2 deficiency-mediated oxidative stress, contributed to the induction of hepatic IR.

Materials and methods

Animals and treatment. Wild-type (WT; n=16) and Nrf2-null (n=16) male mice (6-8 weeks old; weight, 22-24 g) with an ICR background were purchased from the Comparative Medicine Department of Nanjing General Hospital (Nanjing, China). The mice were housed in a specific facility with a 12-h light/dark cycle, temperature maintained at 18-22°C and humidity at 50-60%. The mice were allowed to acclimate to laboratory conditions for 1 week prior to being sacrificed with intraperitoneal anesthetization by 1% pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 50 mg/kg. Subsequently, serum samples, which were obtained following centrifugation at 1,200 x g for 10 min at 4°C, and liver tissue specimens were collected immediately for further examination. The liver was divided as follows: A 1/3 was fixed in an aqueous solution of 4% formaldehyde and the remaining tissue was stored at -80°C. Throughout the investigation, all animal experiments were performed following the Fourth Military Medical University animal use guidelines, and the protocols were approved by the Fourth Military Medical University Animal Care Committee (Xi’an, China).

Determination of liver triglyceride (TG) concentrations. TG concentration in liver tissues was determined by the GPO-PAP method as previously described (16), and was examined enzymatically using commercially available kits according to the manufacturer’s protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histopathology. Liver tissues were fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 24 h, embedded in paraffin, cut into sections ~3x2 cm, stained with hematoxylin for 15 min and subsequently with eosin for 3 min at room temperature. The sections were analyzed under a conventional light microscope (Olympus Corporation, Tokyo, Japan).

Fasting blood glucose (FBG) level determination. FBG levels were determined spectrophotometrically using a commercial kit according to the manufacturer’s protocol (Nanjing Jiancheng Bioengineering Institute).

Intraperitoneal glucose tolerance test (iPGTT). iPGTT was performed to estimate IR as previously described (17). Briefly, following fasting overnight for 12 h, mice were injected intraperitoneally with glucose (20%, 2 g/kg) or insulin (5 U/kg). Glucose level was measured at 0, 15, 30, 60, and 120 min time points from tail-bleed samples and the area under the curve (AUC) for blood glucose was calculated.

Measurement of lipid peroxidation and glutathione (GSH) levels. Lipid peroxidation in the liver was determined by measuring malondialdehyde (MDA) levels using the thiobarbituric acid method (18), and oxidative stress was estimated by measuring GSH levels using a colorimetric assay. Briefly, 500 mg liver tissue was homogenized in 1 ml ice-cold phosphate-buffered saline (PBS) and was centrifuged at 500 x g for 10 min at 4°C. The levels of MDA and GSH in the supernatants were determined, according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from homogenized tissues using TRIzol® reagent according to the manufacturer’s protocol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA concentration was determined using a spectrophotometer at 260 nm. The RT reaction was performed using PrimeScript RT reagent kit (TaKaRa Biotechnology, Co. Ltd., Dalian, China). 1 µg RNA was used in the reaction at 37°C for 15 min, followed by 85°C for 5 sec. qPCR was conducted using a SYBR Premix Ex Taq II kit (Takara Biotechnology, Co. Ltd.) in a 20 µl reaction. The primer sequences used were as follows: TNF-α, forward (F) 5'-CCCAGGCAGTCGACCATCACCTTC-3' and reverse (R) 5'-AGCTGCCCCTCCAGTGT-3'; IL-6, F 5'-GTACTAC CTCTGACCGCAT-3' and R 5'-TGATCTTCGTCGTTCTC-3'; and GAPDH, F 5'-AGGTGGGCTGTAACGGT-3'.
TTG-3' and R 5'-TGTAGACCATGTAGTTAGGTCA-3'. All primers were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, China). The mRNA expression levels of TNF-α and IL-6 were determined using the LightCycler 480 system (Roche Diagnostics, Basel, Switzerland). The thermocycling conditions were as follows: 95°C for 5 min; followed by 45 cycles at 95°C for 10 sec, 58°C for 10 sec and 72°C for 10 sec; and 1 cycle at 95°C for 5 sec, 50°C for 1 min, and finally 40°C for 30 sec. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Relative expression level of TNF-α and IL-6 were calculated with normalization to GAPDH values using the 2^ΔΔCt method (19). All of the reactions were performed in triplicate.

**Results**

**Deletion of Nrf2 initiates obesity and steatohepatitis in HFD-treated mice.** To observe the effect of HFD treatment on mice, their body weight was measured weekly and hepatic weight was measured after 8 weeks. The weight of WT and Nrf2-null mice fed a HFD was significantly increased compared with the control group (P<0.05; Fig. 1A). When fed a HFD, Nrf2-null mice gained more weight than the WT mice; however, there was no statistically significant difference between the two groups (Fig. 1A). Similarly, the weight of hepatic tissues in the Nrf2-null-HFD group was 2.60±0.20 g and was 1.92±0.07 g in the WT-HFD group, both of which were significantly increased in comparison with their control counterparts (P<0.05; Table 1). In addition, hepatic weight in the Nrf2-null-HFD mice was greater than that in the WT-HFD mice (P<0.05; Table 1). Conversely, the WT and Nrf2-null mice fed a control diet experienced no significant increase in body or hepatic weight.

Histopathological analysis was performed and TG concentration was examined in the various groups. Histopathological examination revealed that there was a normal lobular structure and no evidence of inflammation in the livers of mice fed the control diet (Fig. 1B). However, hepatic cells from WT-HFD mice were evidently disordered, with increased microvesicular lipid accumulation and minimal to mild inflammation (Fig. 1B). Furthermore, liver tissue samples from the Nrf2-null-HFD group exhibited disordered hepatic structure, such as severe fat deposition, inflammatory infiltration and hyaline degeneration in hepatic cells (Fig. 1B). However, no fibrosis was observed in any of the groups. The liver TG concentration was 13.16±0.94 mmol/l in the Nrf2-null-HFD mice and 6.50±0.30 mmol/l in the WT-HFD mice, which was significantly higher compared with both control groups (P<0.05; Table 1). Notably, TG concentration in hepatic tissues from Nrf2-null-HFD mice was much higher than that from WT-HFD mice (P<0.05; Table 1). These data indicate that Nrf2 deficiency may promote the development and progression of obesity and steatohepatitis in HFD-treated mice.

**Nrf2 deletion promotes HFD-induced hepatic IR.** To explore the importance of Nrf2 deletion in IR, FBG levels from the blood serum were determined, and an iPGTT was performed in the treatment groups. There was a notable increase in serum FBG levels in the WT mice (4.92±0.18 mmol/l) and Nrf2-null mice (5.60±0.29 mmol/l) fed a HFD compared with mice fed the control diet (P<0.05; Table 1). Although serum FBG levels in the Nrf2-null-HFD mice were slightly higher than in the WT-HFD mice, no significant difference was detected (Table 1). In addition, plasma glucose levels during iPGTT were significantly increased at 15, 30 and 60 min when the HFD-treated groups were compared with the control groups (P<0.05; Fig. 2A). In addition, the AUC of iPGTT, was used to compare impaired glucose tolerance level, and HFD induced a significantly increased area (P<0.05; Fig. 2B). Glucose levels and HFD-induced AUC of iPGTT exhibited greater significant increases in the Nrf2-null group than in the WT group.

The expression levels of proteins involved in the IRS1/PI3K/Akt pathway, which is important for the onset of
IR, were also detected. There was no change in the expression levels of total IRS1 or Akt in liver tissues from any group. However, the levels of p-Tyr-IRS1 and p-Akt were significantly decreased in mice fed a HFD (Fig. 2C). Subsequently, the expression levels of p-GSK-3β and p-FoxO1 in hepatic tissues were determined, since they are key downstream effectors in the IRS1/PI3K/Akt signaling pathway. The levels of p-GSK-3β and p-FoxO1 were notably reduced in HFD groups (Fig. 2D).

Furthermore, Nrf2-null mice exhibited reduced levels of p-GSK-3β and p-FoxO1 compared with WT mice treated with a HFD. These results indicate that Nrf2 deficiency may lead to hepatic IR in mice fed a HFD.

Nrf2 deletion leads to IR by activating oxidative stress in the livers of HFD-treated mice. It has previously been confirmed that oxidative stress is an important factor in IR (11). Therefore, in order to understand the underlying mechanism by which Nrf2 deletion leads to the induction of IR and to investigate if oxidative stress is involved in the process, the concentration of hepatic MDA was determined, as it is one of the products of lipid peroxidation. Hepatic GSH levels were also determined, since GSH is an important endogenous antioxidant, which reduces ROS levels. MDA concentration was significantly enhanced in the livers of WT and Nrf2-null mice following HFD feeding for 8 weeks compared with those fed a control diet. In addition, MDA concentration was significantly greater in Nrf2-null mice compared with in WT mice (P<0.05; Fig. 3A). Conversely, the decrease in GSH levels was greater in the livers of HFD groups compared with those from control groups. In addition, the magnitude of the decrease in GSH concentration was greater in Nrf2-null-HFD mice compared with in WT-HFD mice (P<0.05; Fig. 3B).

The expression levels of a typical Nrf2-regulated gene, Nqo1 were subsequently detected in liver tissues. HFD feeding significantly upregulated the expression levels of nuclear Nrf2 in the livers of WT mice (P<0.05; Fig. 3C), without influencing total protein level. However, neither total nor nuclear Nrf2 expression was observed in the Nrf2-null mice fed any diet. Furthermore, the protein expression levels of Nqo1 were significantly downregulated in the livers of Nrf2-null-control mice compared with in the WT-control mice (P<0.05; Fig. 3C). The protein expression levels of Nqo1 were also significantly upregulated by HFD feeding in WT mice but not in Nrf2-null mice (P<0.05; Fig. 3C).

Nrf2 deficiency exacerbates activation of NF-κB in the livers of HFD-treated mice. The IKK/NF-κB signaling pathway may be activated by oxidative products, including MDA and ROS.
and is associated with oxidative stress. The release of TNF-α and IL-6 following activation of the IKK/NF-κB signaling pathway has been demonstrated to be associated with the pathogenesis of IR by interfering with the IRS1/PI3K/Akt signaling pathway (21). Therefore, the expression levels of TNF-α and IL-6 were detected in the livers of the different treatment groups using RT-qPCR and ELISA. HFD feeding significantly enhanced the mRNA expression levels of the two cytokines (21).

Figure 2. Nrf2 deletion led to insulin resistance when mice were subjected to a HFD. (A) Blood glucose levels at 0, 15, 30, 60 and 120 min were determined following injection with glucose (2 g/kg body weight) in the four groups using ipGTT. *P<0.05, Nrf2-null + HFD vs. Nrf2-null mice groups; **P<0.05, WT + HFD vs WT mice groups. (B) AUC was calculated from the results of the ipGTT at 15 min. Expression levels of (C) total IRS1, p-Tyr IRS1, total Akt and p-Akt (Ser473), (D) p-GSK-3β and p-FoXO1 in liver tissues was examined using western blot analysis. *P<0.05. Data are presented as the mean ± standard error. WT, wild-type; Nrf2, nuclear erythroid 2-related factor 2; HFD, high-fat diet; AUC, area under curve; IRS, insulin receptor substrate 1; p-GSK-3β, phosphorylated-glycogen synthase kinase 3β; p-FoXO1, phosphorylated-forkhead box O1; ipGTT, intraperitoneal glucose tolerance test.
compared with the control group (P<0.05; Fig. 4A and B). In addition, the mRNA expression levels of TNF-α and IL-6 were significantly higher in the livers of Nrf2-null mice compared with those of WT mice fed a HFD (P<0.05; Fig. 4A and B). The protein expression levels of TNF-α and IL-6 were also correlated with the mRNA levels (P<0.05; Fig. 4C and D). Subsequently, the expression levels of the upstream regulator of TNF-α and IL-6, NF-κB, were determined in the livers of HFD-treated mice. The total protein levels of NF-κB were not significantly different between groups. However, the expression levels of nuclear NF-κB were significantly upregulated in both WT-HFD and Nrf2-null-HFD mice (P<0.05; Fig. 4E), the magnitude of upregulation was greater in Nrf2-null mice compared with WT mice (P<0.05; Fig. 4E). These results indicate that Nrf2 deletion may induce hepatic IR by activating the NF-κB signaling pathway in HFD-treated mice.

Discussion

NAFLD, which is associated with hepatic cirrhosis and liver-related mortality, is a major public health issue (22). Through extensive previous research, it has become clear that oxidative stress, inflammation and IR contribute to the pathogenesis of NAFLD (23), and several signaling pathways are involved in the process. The present study determined that deletion of the Nrf2 gene may lead to mice being more susceptible to the development of NAFLD, partially due to hepatic IR induced by a HFD.

Nrf2 is a member of the basic leucine zipper transcription factor family, which mediates the expression of a series of antioxidant and detoxification genes (24). It is expressed in various organs, including the liver, lungs, kidneys, digestive tract and fat tissue (25,26). It has previously been demonstrated that Nrf2 is important for the onset of NAFLD alongside the administration of various diets, and Nrf2-null mice have exhibited rapid progression of steatohepatitis when fed an atherogenic HFD (27). In addition, Sugimoto et al (28) and Chowdhry et al (15) demonstrated that limitation of Nrf2 activity significantly promoted the progression of NASH, from NAFLD to NASH, in mice placed on an MCD diet. In the present study, a HFD was able to induce weight gain of body and liver tissues in both WT and Nrf2-null mice compared
with the control diet. Furthermore, the increase observed was greater in the livers of Nrf2-null mice compared with WT mice. TG accumulation in the hepatic tissues of Nrf2-null mice fed a HFD was also greater when compared with the control mice, which led to alterations in liver structure accompanied by fat deposition, inflammatory infiltration and hyaline degeneration.

IR has been identified as a major pathophysiological feature in several metabolic diseases, including NAFLD (29). A previous study demonstrated that hepatic IR may result in the development of metabolic dyslipidemia and hepatic steatosis in AKT2-mutant individuals (30). Furthermore, another previous study using tissue-specific knockout analysis demonstrated that knockdown of hepatic insulin receptors may lead to IR, severe glucose intolerance, and liver steatosis with a high level of fasting hyperglycemia and postprandial blood glucose in mice (31). The process of IR is usually induced by various factors, including genetic determinants, nutrition and lifestyle. However, it has previously been demonstrated that oxidative stress and inflammation are associated with hepatic IR (32). The insulin signaling pathway is of pivotal importance. Subsequent to binding insulin, the insulin receptor is activated and autophosphorylated, resulting in IRS phosphorylation of tyrosine residues (33). In response to IRS phosphorylation, various downstream signaling molecules, such as the PI3K/AKT or mitogen-activated protein kinase pathways, are activated. In the majority of cases of IR, the aforementioned molecular event is often impaired (31). The present study determined that both serum FBG levels and plasma glucose levels during iPGTT were significantly increased in mice following administration of a HFD for 8 weeks, particularly in the Nrf2-null-HFD group. Furthermore, the expression levels of phosphorylated proteins associated with the IRS1/PI3K/Akt signaling pathway were reduced in the livers of Nrf2-null mice fed a HFD. These results indicated that Nrf2 deletion may lead to hepatic IR in HFD models.

**Figure 4.** Nrf2 deletion activated the NF-κB signaling pathway in mice fed a HFD. mRNA expression levels of (A) IL-6 and (B) TNF-α in liver tissues of WT and Nrf2-null mice fed a control diet or HFD were evaluated by reverse transcription-quantitative polymerase chain reaction. Protein levels of (C) IL-6 and (D) TNF-α in liver tissues of the four groups were evaluated by enzyme-linked immunosorbeant assay. (E) Total and nuclear NF-κB levels expressed in liver tissues of WT and Nrf2-null mice were evaluated by western blot analysis. Data are presented as the mean ± standard error. *P<0.05. IL-6, interleukin-6; Nrf2, nuclear erythroid 2-related factor 2; WT, wild-type; HFD, high-fat diet; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor-κB.
NF-kB is a primary transcription factor in inflammatory diseases (34), which also contributes to the pathology of IR (35). Activation of the hepatic NF-kB pathway has been suggested to be directly responsible for HFD-induced IR (36). Inactivated NF-kB in the cytosol binds to inhibitor of kB (IkB) molecules and its transcriptional function is prevented. When the IKK is activated by extracellular stimuli or products of oxidative stress it phosphorylates IkB, promoting the detachment of NF-kB from the IKK complex and resulting in nuclear translocation (37). The activation of NF-kB consequently leads to vast increases in the production of inflammatory cytokines, including IL-6, TNF-α and IL-1β, which are considered pathogenetic markers that have a pivotal role in IR (32). The present study confirmed that Nrfl2 deficiency may increase hepatic MDA levels and reduce GSH levels in mice fed a HFD. The Nrfl2-null-HFD mice suffered IR through the activation of NF-kB and its downstream cytokines IL-6 and TNF-α.

The present study demonstrated that Nrfl2 deficiency may induce obesity, hepatic IR and alterations in liver tissue structure in mice fed a HFD. Hepatic IR induced by Nrfl2 deletion is regulated by activation of the NF-kB signaling pathway, which is associated with oxidative stress, as determined by increased hepatic MDA concentrations and decreased GSH levels. However, further investigations are required to clarify whether other mechanisms are involved in IR in NAFLD mediated by Nrfl2 deletion. The findings of the present study present novel insights into the mechanisms underlying IR and NAFLD, and a potential therapeutic strategy for future treatment.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 30800515, 81270485 and 81170376) and the Natural Science Foundation of Shaanxi Province (grant no. 2013JM4021).

References

26. Chan K, Lu R, Chang JC and Kan YW: NRF2, a member of the NF-E2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci USA 93: 13943-13948, 1996.