

Non-esterified fatty acids activate the ROS–p38–p53/Nrf2 signaling pathway to induce bovine hepatocyte apoptosis in vitro

Yuxiang Song · Xinwei Li · Yu Li · Na Li ·
Xiaoxia Shi · Hongyan Ding · Yuhang Zhang ·
Xiaobing Li · Guowen Liu · Zhe Wang

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Abstract A high plasma concentration of non-esterified fatty acids (NEFAs) is an important pathogenic factor that leads to ketosis and fatty liver in dairy cows. NEFAs may be associated with oxidative stress in dairy cows with ketosis or fatty liver and the subsequent induction of hepatocyte damage. However, the molecular mechanism of NEFAs-induced oxidative stress and whether NEFAs cause apoptosis of hepatocytes are unclear. Therefore, the aim of this study was to investigate the molecular mechanism of NEFAs-induced oxidative liver damage in bovine hepatocytes. The results showed that NEFAs increased oxidative stress, resulting in p38 phosphorylation. High activated p38 increased the expression, nuclear localization and transcriptional activity of p53 and decreased the nuclear localization and transcriptional activity of Nrf2 in bovine hepatocytes treated with high concentrations of NEFAs. High concentrations of NEFAs also promoted the apoptosis of bovine hepatocytes. Both *N*-acetyl-L-cysteine (NAC)

and glucose (GLU) could attenuate the NEFA-induced apoptotic damage. These results indicate that NEFAs activate the ROS–p38–p53/Nrf2 signaling pathway to induce apoptotic damage in bovine hepatocytes.

Keywords Non-esterified fatty acids · Oxidative stress · Signal pathway · Apoptosis

Abbreviations

BSA	Bovine serum albumin
CAT	Catalase
GLU	Glucose
GSH	Glutathione
GSSG	Glutathione disulfide
GSH-Px	Glutathione peroxidase
Keap1	Kelch-like ECH-associated protein 1
MDA	Malonaldehyde
Mdm2	Mouse double minute 2 homolog
NAC	<i>N</i> -acetyl-L-cysteine
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
Nrf2	Nuclear factor erythroid 2-related factor2
p38MAPK	p38 mitogen-activated protein kinases
p53	Tumor protein p53
RET	Reverse electron transfer
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAC	Total antioxidant capacity

Yuxiang Song and Xinwei Li contributed equally to this study.

Y. Song · X. Li · Y. Li · X. Shi · H. Ding · X. Li (✉) ·
G. Liu (✉) · Z. Wang
Key Laboratory of Zoonosis, Ministry of Education, College
of Veterinary Medicine, Jilin University, 5333 Xi'an Road,
Changchun 130062, Jilin, China
e-mail: xbli@jlu.edu.cn

G. Liu
e-mail: liuguowen2008@163.com

N. Li
Laboratory of Microbiology and Immunology, College of
Veterinary Medicine, Jilin University, Changchun, Jilin, China

Y. Zhang
College of Animal Science and Technology, Jilin Agricultural
Science and Technology College, Jilin, Jilin, China

Introduction

During the transition period, dairy cows experience a state of negative energy balance (NEB) that is induced by a low intake of dry matter and an increased demand for glucose

to support milk production [1]. NEB initiates fat mobilization and a subsequent increase in the blood non-esterified fatty acids (NEFAs) concentration. Investigations have shown that physiological, metabolic and endocrine adaptations occur in the liver to support lipid mobilization during the transition period [2]. Large quantities of NEFAs are metabolized into ketones or synthesized into triglycerides (TG) in hepatocytes, thereby inducing ketosis or fatty liver [3]. Therefore, a high NEFA concentration is an important pathogenic factor and may be associated with the pathogenicity of some NEB-related metabolic disorders, such as ketosis and fatty liver [4]. The NEFAs level is mainly dependent on the degree of fat mobilization. In the late lactation and dry periods in cows, the average plasma NEFAs level is below 0.2 mM. However, if ketosis develops, NEFAs levels will exceed 1.0 mM [5]. The lipotoxicity of NEFAs is mainly due to the production of reactive oxygen species (ROS) during mitochondrial oxidation [6]. Continuous, incomplete NEFAs oxidation results in a high metabolic state and a large number of oxygen radicals in the liver, which induce hepatocyte damage.

NEFAs can regulate mitochondrial ROS generation through a variety of mechanisms, including reducing the activity of antioxidant enzymes, affecting electron transport in the respiratory chain and increasing the fluidity of the mitochondrial membrane [7]. ROS generated by NEFAs will induce secondary “hits” to the liver and cause hepatocyte apoptosis [8]. Abundant evidence demonstrates a relationship between NEFAs and apoptosis [9, 10].

p38 MAP kinase, one of the three MAPK subgroups, plays an important role in signal transduction and biological processes. Several studies have shown that p38 MAPK can be activated by ROS that are generated intracellularly [11]. ROS have been reported to play a critical role in cytokine-induced p38 α activation, and p38 MAPK can function as a sensor of oxidative stress [12]. The activation of p38 MAPK affects the regulation of downstream transcription factors, such as p53 and Nrf2, to control downstream pro-apoptotic and anti-apoptotic gene expression in response to many extracellular stimuli, including oxidative stress [13–15].

p53 is a multifunctional protein that is at the crossroads between DNA damage and apoptosis and has an important role in controlling cellular responses to many stress signals. p53 is normally expressed at low levels and is unable to bind specifically to DNA [16]. Under conditions of stress, p53 accumulates via multiple mechanisms, including enhanced translation, decreased proteolytic degradation and post-translational modification [17]. In addition, p53 is involved in the apoptosis caused by NEFAs in β -cells and human hepatocytes in non-alcoholic fatty liver disease [18, 19]. NF-E2-related factor 2 (Nrf2) is also a key regulatory

factor that can respond to oxidative stress. Nrf2 is anchored to the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm [20]. However, during oxidative stress, Nrf2 is released from the Keap-1/Nrf2 complex to promote the nuclear translocation of Nrf2 and activate ARE-mediated transcription of antioxidant genes such as HO-1, GST and GCS [20]. The relationship between ROS, Nrf2 and p38 is cell type- and stimulus-dependent, but how this relationship affects the transition period in cows with high plasma NEFAs concentrations is unclear.

Dairy cows with ketosis exhibit high blood NEFAs concentrations and oxidative stress. A high plasma concentration of NEFAs is an important pathogenic factor for ketosis and fatty liver in dairy cows. Oxidative stress in dairy cows with ketosis or fatty liver may be associated with high blood NEFAs concentrations. The p38 MAPK-mediated p53 and Nrf2 pathways are involved in oxidative stress-induced cell apoptosis. Therefore, we hypothesized that NEFAs could modulate the ROS–p38–p53/Nrf2 signaling pathway to induce apoptotic damage to bovine hepatocytes. The aim of this study was to investigate the molecular mechanism of NEFAs-induced liver oxidative damage.

Materials and methods

Hepatocyte culture

This study protocol was approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China). The hepatocytes were isolated using a two-step perfusion method published previously [21]. Briefly, the liver caudate lobe was removed from a newborn female Holstein calf after anesthesia, and heparin was injected into the jugular vein. The liver was perfused with perfusion solution for washing and with digestion solution to digest the tissue. Then, we removed the liver capsule, blood vessels, fat and connective tissue. The remainder of the liver parenchyma was cut into pieces and filtered sequentially with cell sieves. The cell density was adjusted to 1×10^6 cells/mL, and the hepatocyte suspension was seeded into a 6-well tissue culture plate (2 mL per well) or a 24-well tissue culture plate (1×10^5 cells/mL, 0.5 mL) and cultured at 37 °C in 5 % CO₂. After 4 h, the medium was replaced with growth medium containing 10 % fetal bovine serum. The medium was replaced with fresh medium every 24 h.

NEFAs preparation and treatment

The composition and concentration of NEFAs used in this study were chosen according to the normal and

pathological hematology standards for dairy cows with or without ketosis. The NEFAs composition included oleic acid (2.175 mM), linoleic acid (0.245 mM), palmitic acid (1.595 mM), stearic acid (0.72 mM) and palmitoleic acid (0.265 mM) adjusted to pH 7.6 with hydrochloric acid (1 mM).

The hepatocytes were subjected to the following treatments after 72 h of culture [21]. Before NEFAs treatment, the cells were cultured overnight without serum. The concentration of bovine serum albumin (BSA) in the cell culture medium was 3.8 %. To identify the proper time point for the time gradient experiments, hepatocytes were treated with 2.4 mM NEFAs for 0, 0.5, 1, 3, 6, 9, 12 and 24 h. For the dose response experiments, the hepatocytes were treated with 0, 0.6, 1.2 and 2.4 mM NEFAs (with or without SB203580, NAC and glucose) for 9 h.

Apoptosis assay

Apoptosis was detected in hepatocytes after treatment with NEFAs. In brief, hepatocytes were treated with 0, 0.6, 1.2 and 2.4 mM NEFAs (with or without SB203580, NAC and glucose). The hepatocytes were stained with Annexin V-FITC/PI (BD Biosciences, San Jose, CA, USA) before detection by fluorescence microscopy and flow cytometry. The hepatocytes were digested with trypsin after treatment with NEFAs for 24 h. DNA was collected according to the protocol of the DNA Ladder Extraction Kit (Beyotime Biotechnology Inc., Shanghai, China). The extracted DNA was resolved by electrophoresis at 80 V for 1 h.

Western blotting assay

The hepatocyte total proteins and nuclear proteins were extracted according to the manufacturer's instructions (Sangon Biotech Co., Ltd, Shanghai, China). The target proteins were separated by polyacrylamide gel electrophoresis and then electrotransferred onto PVDF membranes. The membranes were blocked with 5 % BSA in TBST buffer for 2 h at 4 °C and hybridized with specific antibodies, including antibodies to cleaved Caspase 3 and p-p38 from Cell Signaling Technology (Danvers, MA, USA); Caspase 9, Bax and Nrf2 from Santa Cruz Biotechnology (Santa Cruz, CA); and Caspase 3, p38, cleaved PARP, Bcl-2 and p53 from Abcam (Cambridge, MA, USA). The membranes were then incubated with a secondary antibody. Immunoreactive bands were detected with enhanced chemiluminescence solution (ECL, Beyotime Biotechnology Inc., China). The bands were detected and analyzed using a Protein Simple Imager.

p38a Enzyme activity detection assay

p38 α enzyme activity was detected in hepatocytes after treatment with NEFAs for 9 h using a p38 α kinase activity spectrophotometric quantification kit (GENMED Scientifics, INC, USA). The hepatocytes were lysed using GENMED lysis buffer, vortexed for 15 s, incubated in an ice bath for 30 min and centrifuged at 1,600 g for 5 min at 4 °C. The absorbance of the supernatant was detected using a spectrophotometer (ELX 800, BIO-TEK, USA).

Determination of intracellular ROS concentration and oxidative stress indices

After treatment with NEFAs for 9 h, the hepatocytes were incubated with dichlorofluorescein diacetate (DCFH-DA) (Beyotime Biotechnology Inc., China) for 30 min at 37 °C. The fluorescence intensity of cells was measured by flow cytometry (Becton–Dickinson, Franklin Lakes, NJ, USA). The total antioxidant capacity (TAC), malondialdehyde (MDA), glutathione (GSH) and glutathione disulfide (GSSG) content, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were measured according to the manufacturer's instructions (Beyotime Biotechnology Inc., China).

Real-time qRT-PCR assay

The total RNA from hepatocytes was extracted using Trizol (TaKaRa Biotechnology Co., Ltd.). RNA quality was assessed by electrophoresis (1 % agarose gels) before reverse transcription to form cDNA (TaKaRa Biotechnology Co., Ltd.). The primers of the genes were designed using Primer Express software (PE Applied Biosystems, Inc., Foster City, CA, USA) and are shown in Table 1. The mRNA expression levels were evaluated by qRT-PCR using the SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co., Ltd.) and the 7000 Fast Real-Time PCR System (Applied Biosystems). The relative expression of each gene was normalized to β -actin.

Immunofluorescence assay

Hepatocytes were treated with NEFAs for 9 h. The hepatocytes were fixed with 10 % formalin in 0.1 % PBS for 20 min. Antigen retrieval was performed using EDTA-Na₂ at 95 °C for 5 min. The hepatocytes were permeabilized using 0.1 % Triton X-100 and incubated with specific antibodies overnight at 4 °C. Next, the hepatocytes were incubated with secondary antibodies for 30 min. Their nuclei were stained with Hoechst 33258. Finally, the coverslips were sealed with glycerol, and the samples were observed by laser confocal microscopy (FV500, OLYMPUS, China).

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was used to detect the transcriptional activity of p53 and Nrf2. Nuclear proteins were extracted using a nuclear protein extraction kit (Sangon Biotech Co., Ltd, Shanghai, China). The special probe recognition sequences are 5' TACAG AACATGTCTAAGCATGCTGGGGACT 3' for p53 and 5' ACTGAGGGTGACTCAGCAAAAATC 3' for Nrf2. The probes were labeled with biotin by incubating with the label for 30 min at 37 °C. The binding reaction was performed using the Lightshift EMSA Optimization and Control Kit (Pierce Biotechnology, Inc., Rockford, USA). The nuclear proteins were collected after NEFAs treatment for 9 h. The DNA–protein complexes were separated by electrophoresis with non-denatured 6.5 % polyacrylamide TBE gels and electrotransferred onto a nylon membrane that was crosslinked using a UV light crosslinker (Cany Precision Instruments Co., Ltd., Shanghai, China). The biotin-labeled probe was detected using chemiluminescence solution (Pierce Biotechnology, Inc., Rockford, IL, USA). After exposing the blots to X-ray film, we measured the band intensity using a LAS3000 Bioimage analyzer (Fuji Photo Film Co., Beijing, China).

Results

NEFAs induce hepatocyte apoptosis through oxidative stress

NEFAs can cause oxidative stress in hepatocytes. High concentrations of NEFAs decreased the TAC content and

Fig. 1 NEFAs induce oxidative stress in hepatocytes. The hepatocytes were treated with NEFAs for 24 h. **A** The MDA and the TAC content, and the GSH-Px, CAT and SOD activities. * $p < 0.05$; ** $p < 0.01$ versus the control group (0 mM NEFAs). **B** The GSH, GSSG contents and the GSH/GSSG rates. * $p < 0.05$; ** $p < 0.01$ versus the control group (0 mM NEFAs). **C** ROS levels were detected by flow cytometry. * $p < 0.05$; ** $p < 0.01$ versus the control group (0 mM NEFAs). *Open circles* indicate a significant difference

the activity of the GSH-Px, CAT and SOD while increasing the MDA content in hepatocytes (Fig. 1A). At the same time, high concentrations of NEFAs decreased GSH and increased GSSG, as a result, the rate of GSH/GSSG decreased in high concentrations of NEFAs groups (Fig. 1B). Relative to the control group (0 mM), the 1.2 and 2.4 mM groups had markedly elevated ROS levels, and GLU was able to attenuate this effect (Fig. 1C). The hepatocyte apoptotic rate was detected and the results showed that the apoptotic rate increased in the 1.2 and 2.4 mM NEFAs groups but was inhibited by NAC or GLU (Fig. 2a). The apoptosis levels from DNA ladder results were consistent with those from fluorescence microscopy (Fig. 2b). Altogether, these results indicate that high concentrations of NEFAs induce hepatocyte apoptosis by causing oxidative stress.

NEFAs induced hepatocyte apoptosis mediated by the ROS–p38 signaling pathway

The p38 signaling pathway is implicated in the hepatocyte apoptosis caused by NEFAs

We detected the activity and mRNA expression of p38 and found that NEFAs increased p38 activity (Fig. 3Aa) as well

Table 1 The primer sequences of designated genes

Gene	Sequence number	Primer sequences (5'–3')	Length (bp)
β-Actin	BC 142413.1	For GCCCTGAGGCTCTCTTCCA Rev GCGGATGTCGACGTCACA	101
p38 MAPK	NM_001102174.1	For AAGTAGCCAGGTTGTCTG Rev AGAGGAATGGCGATGA	156
Caspase 3	NM_001077840	For AGAACTTGGTGGTCCATACA Rev CTCAACCCGTCTCCCTTTAT	242
Caspase 9	NM_001205504.1	For TTCCCCCTTATTTTGCTC Rev ACGGCTCCCACCCAGTC	148
Nrf2	NM_001011678	For GCCCTCACTGGATAAAGAA Rev CATGCCGTTGCTGGTAC	202
p53	NM_174201.2	For GAGCACTGCCTACCAACA Rev CATCCAGAGCATCCTTCA	150
Bax	XM_004767368.1	For CCTTTTGCTTCAGGGTTTC Rev GCTCAGCTTCTTGGTGGAT	109
Bcl-2	NM_001166486	For TGACCGAGTACCTGAACCG Rev CAGCCAGGAGAAATCAAACA	116

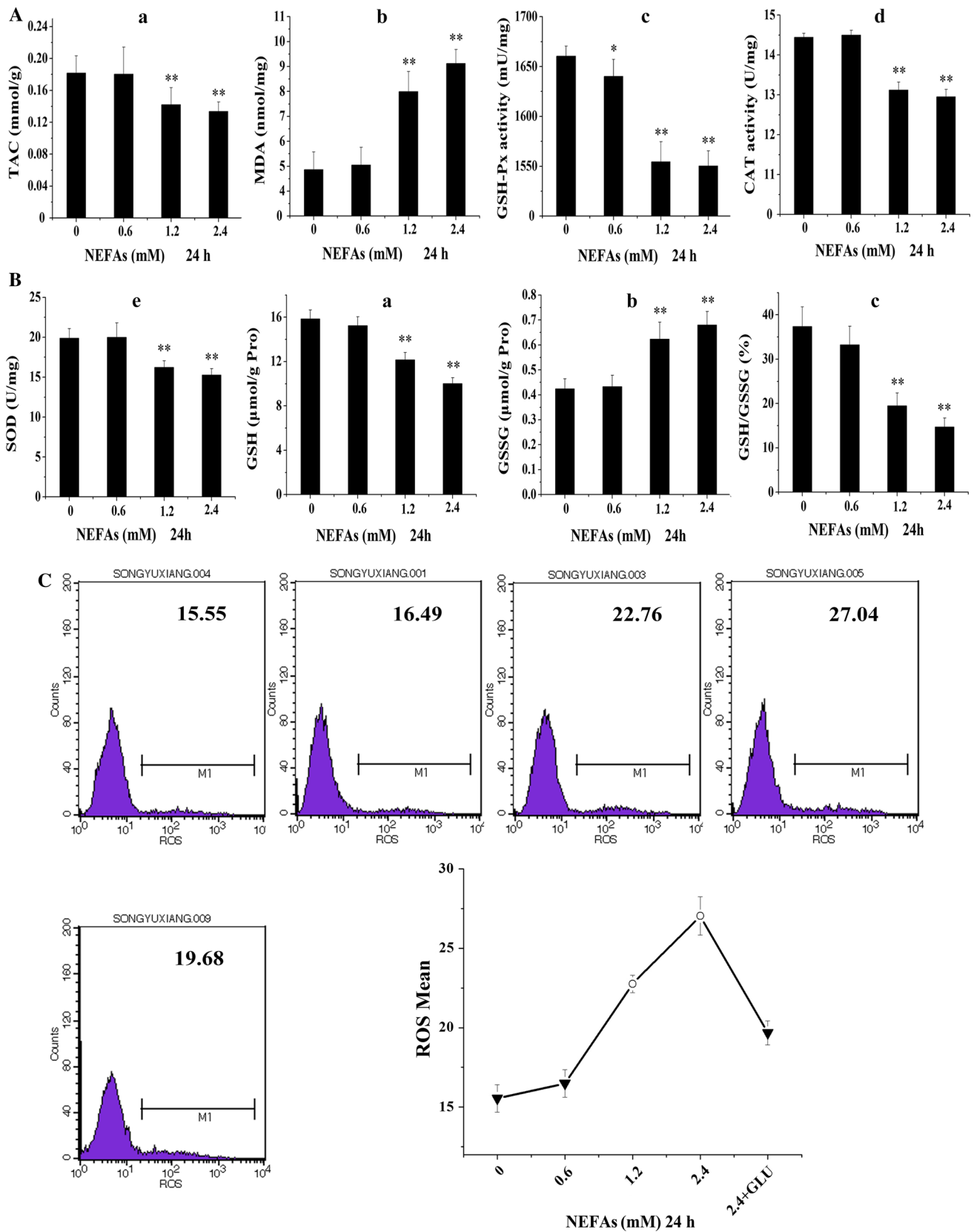
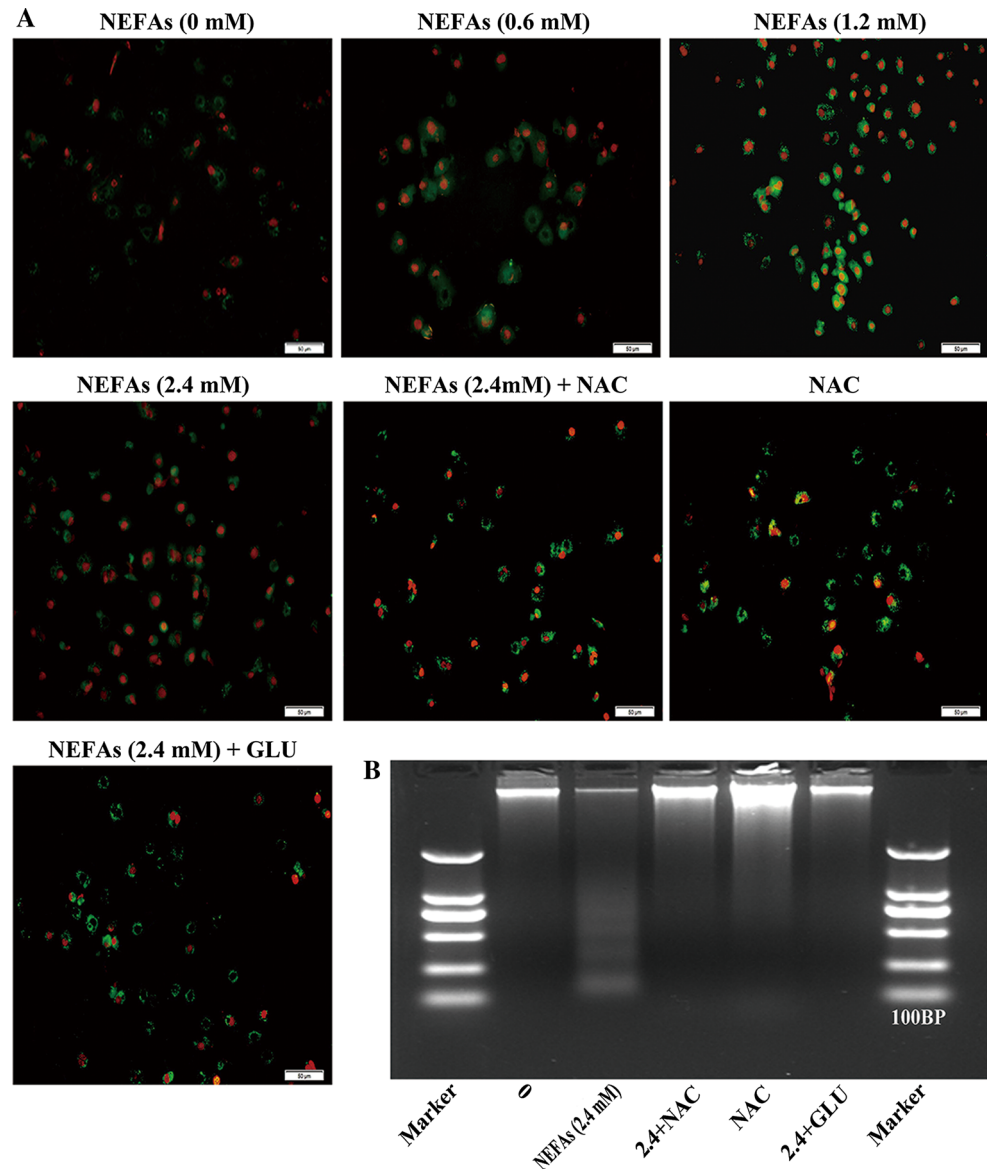


Fig. 2 NEFAs induce oxidative stress and subsequent hepatocyte apoptosis. The hepatocytes were treated with NEFAs, NEFAs + NAC and NEFAs + GLU for 24 h. **a** Apoptotic hepatocytes observed under a fluorescence microscopy stained with annexin v-FITC/PI respectively. **b** Hepatocytes apoptosis were analyzed by DNA ladder respectively



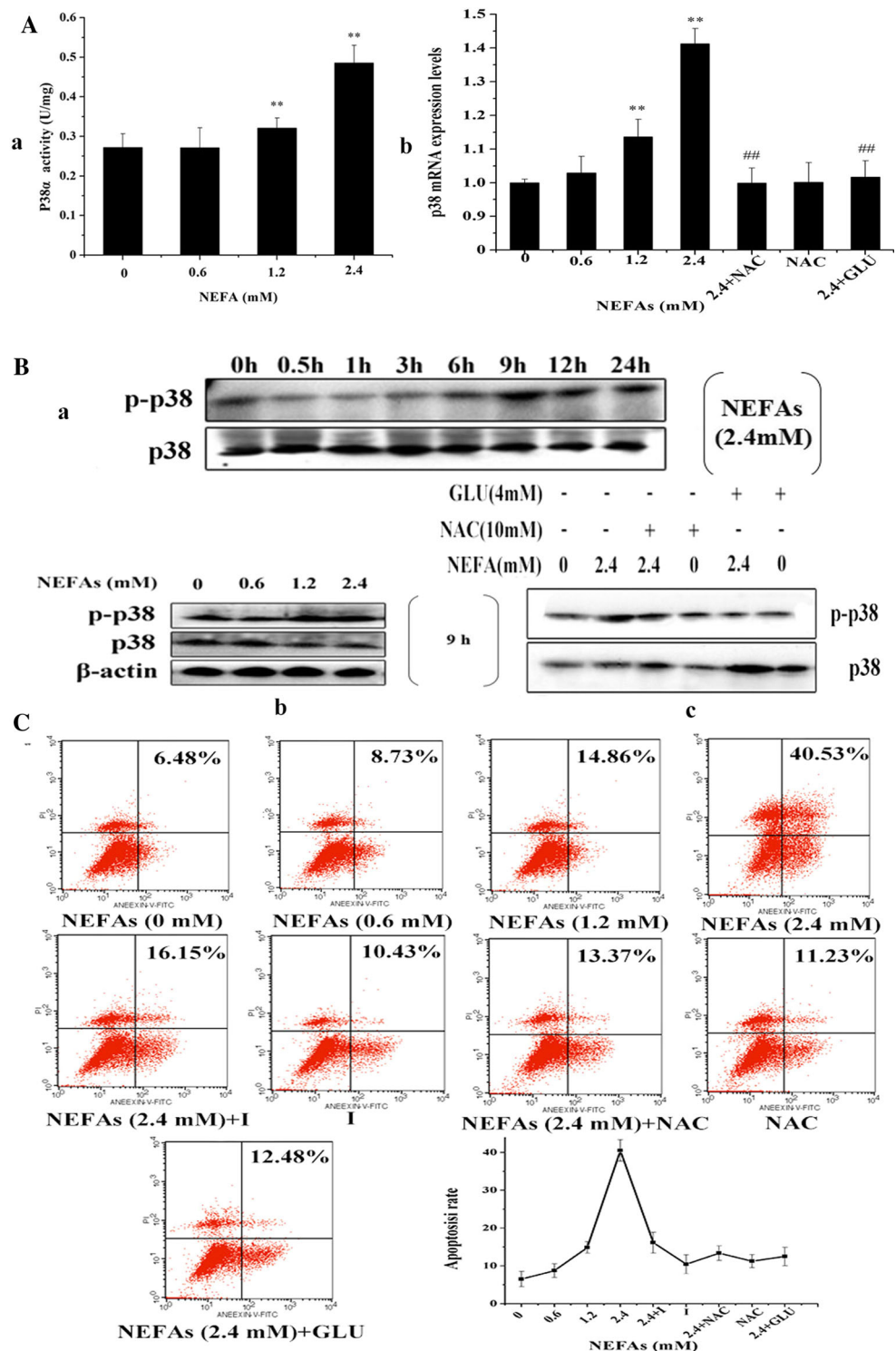
as the mRNA expression of p38 (Fig. 3Ab) in a dose-dependent manner. However, p38 activity and mRNA expression decreased significantly in the NAC and GLU treatment groups ($p < 0.01$) (Fig. 3Ab). These results indicate that p38 plays an important role in the peroxidative damage to hepatocytes caused by NEFAs. Time course experiments for p38 activation were performed with NEFAs (2.4 mM), and the results showed that the phosphorylation levels of p38 peaked at 9 h (Fig. 3Ba). Western blot results showed that p38 was activated by NEFAs in a dose-dependent manner (Fig. 3Bb) and that activation was low in the NAC and GLU treatment groups (Fig. 3Bc). An investigation of the apoptotic rate of hepatocytes revealed that NEFAs caused hepatocyte apoptosis in a dose-dependent manner, and both NAC and GLU protected hepatocytes against apoptosis caused by NEFAs (Fig. 3C).

These results indicate that the p38 signaling pathway is involved in the hepatocyte apoptosis caused by NEFAs.

The p38 pathway mediated NEFAs-induced hepatocyte apoptosis

Bcl-2 family members play a vital role in the regulation of cell apoptosis. Therefore, we detected the levels of Bax, Bcl-2, Caspase 3 and Caspase 9. Relative to the control group, the mRNA levels of Bax, Caspase 3 and Caspase 9 were increased in the 1.2 and 2.4 mM NEFAs groups, whereas Bcl-2 decreased significantly in the 2.4 mM NEFAs group (Fig. 4A). As shown in Fig. 4Ba and c, NEFAs increased the protein expression of Caspase 9 and Bax and promoted the decomposition and activation of Caspase 3 and PARP, whereas the protein expression levels of Bcl-2 were

Fig. 3 The p38 signaling pathway is implicated in hepatocyte apoptosis caused by NEFAs. The hepatocytes were treated with NEFAs for either 9 or 24 h. **A** The activity and mRNA expression levels of p38. **B** The time course and concentration experiments for p38 activation. **C** The apoptotic rates of hepatocytes detected by flow cytometry. * $p < 0.05$; ** $p < 0.01$ versus the control group (NEFAs 0 mM). # $p < 0.05$; ## $p < 0.01$ versus the 2.4 mM NEFAs group. Open circles indicate a significant difference

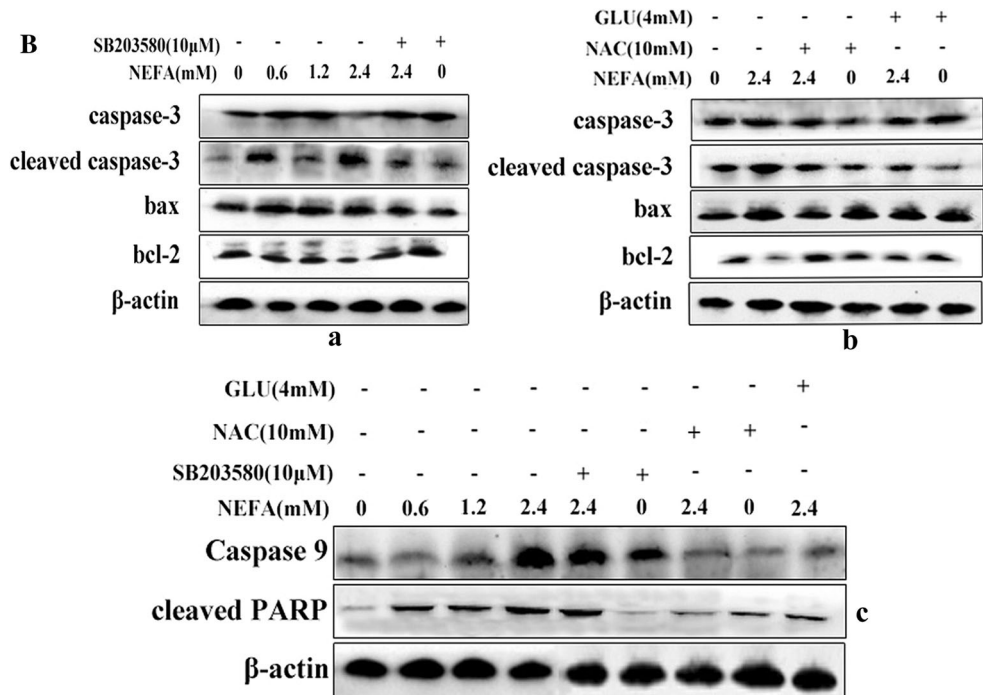
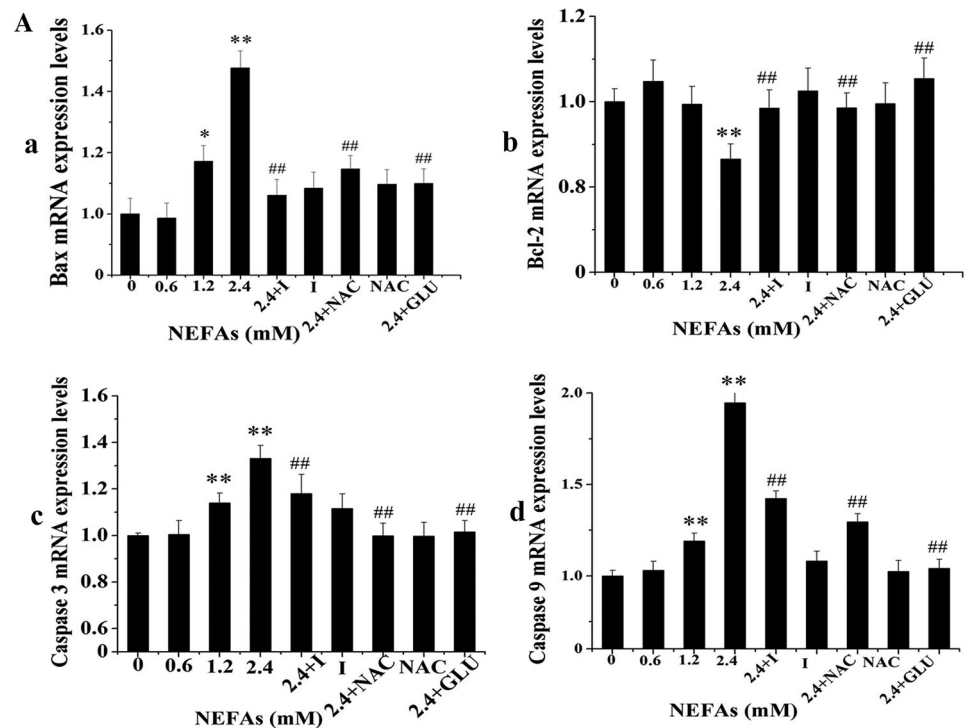


decreased. The SB203580 (Selleck Chemicals LLC, Houston, TX, USA), NAC (Sigma-Aldrich, St Louis, MO, USA) and GLU (Sigma-Aldrich, St Louis, MO, USA) groups were resistant to these NEFAs-induced effects at both the transcriptional and translational levels (Fig. 4A, Bb, Bc). These results indicate that NEFAs-induced apoptosis in hepatocytes is mediated by the p38 pathway.

p53 and Nrf2 are involved in p38-mediated hepatocyte apoptosis caused by NEFAs

p53 is a pivotal regulation center of apoptosis that can be activated by p38. The transcription and nuclear protein levels of p53 were elevated in a NEFAs dose-dependent manner (Fig. 5a, b). Relative to the 2.4 mM NEFAs treatment group,

Fig. 4 NEFAs-induced hepatocyte apoptosis is mediated by the p38 pathway. The hepatocytes were treated with NEFAs for 9 h. **A** The mRNA expression levels of the apoptosis-related genes Bax, Bcl-2, Caspase 3 and Caspase 9. **B** The protein expression levels of Caspase 3, Caspase 9 and Bax, and the activation of apoptosis-related proteins cleaved Caspase 3 and cleaved PARP. * $p < 0.05$; ** $p < 0.01$ versus the control group (NEFAs 0 mM). # $p < 0.05$; ## $p < 0.01$ versus the 2.4 mM NEFAs group

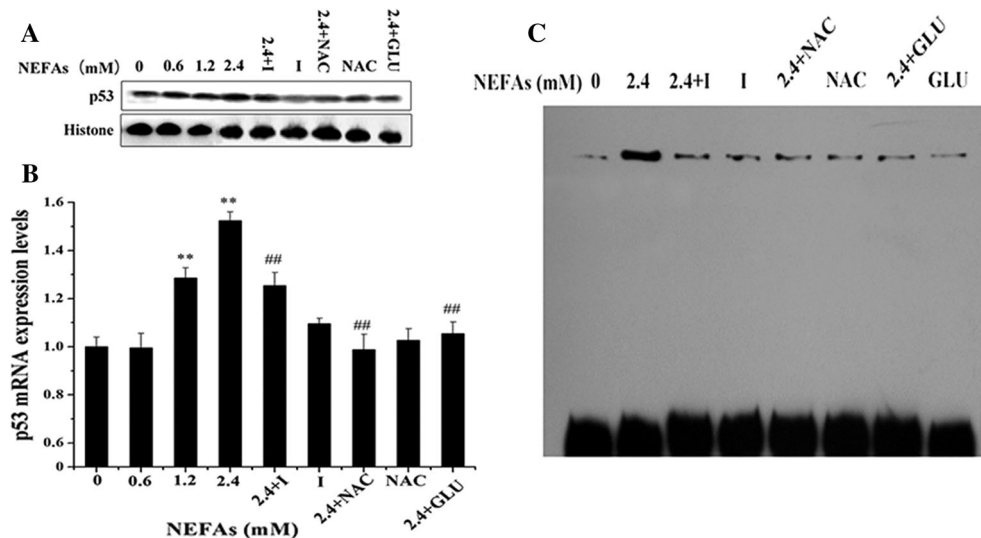


groups treated with SB203580, NAC and GLU had decreased nuclear p53 protein levels (Fig. 5a, b). The transcriptional activity and cellular localization of p53 were detected by EMSA and IF assays, respectively. Relative to the control group, NEFAs increased p53 transcriptional activity and promoted translocation to the nucleus. SB203580, NAC and GLU decreased the transcriptional

activity and the levels of nuclear translocation (Figs. 5c, 6). Collectively, these results suggest that NEFAs activate p38 and then increase the expression and transcriptional activity of p53, which promotes hepatocyte apoptosis.

Nrf2 is an important transcription factor that is closely associated with cell antioxidants and that regulates the expression of many antioxidases. Nrf2 also has a complex

Fig. 5 The effects of NEFAs on the expression and transcriptional activity of p53 in bovine hepatocytes. The hepatocytes were treated with NEFAs for 9 h. **a** Nuclear p53 protein levels detected by western blot. **b** p53 mRNA expression levels detected by RT-PCR. **c** The transcriptional activity of nuclear p53 detected by EMSA. * $p < 0.05$; ** $p < 0.01$ versus the control group (NEFAs 0 mM). # $p < 0.05$; ## $p < 0.01$ versus the 2.4 mM NEFAs group



relationship with p38. Compared with the control group, the 1.2 mM NEFAs treatment group had elevated Nrf2 mRNA and nuclear protein levels. However, the nuclear protein levels significantly decreased in the 2.4 mM NEFAs group (Fig. 7a, c). Compared with the 2.4 mM NEFAs group, the GLU treatment group had increased transcriptional activity of Nrf2 but decreased Nrf2 mRNA expression (Fig. 7a, c). The nuclear protein level of Nrf2 increased significantly in the SB203580 treatment group, but the Nrf2 mRNA expression decreased significantly (Fig. 7a, c). Both mRNA and nuclear protein levels of Nrf2 were decreased in the NAC groups (Fig. 7a, c). The transcriptional activity and cellular localization of Nrf2 were detected by EMSA and IF assays, respectively. The results are similar to the Nrf2 western blot results (Figs. 7b, 8) and suggest that high levels of NEFAs (2.4 mM) activate p38, thus, inhibiting the translocation of Nrf2 to the nucleus and the transcriptional activity of Nrf2.

Discussion

Cows experience a series of nutritional, physiological and behavioral changes and become more susceptible to nutritional and metabolic diseases, such as fatty liver and ketosis, during the transition period [22, 23]. These factors, as well as changes in hormone levels, can cause increased fat mobilization and plasma NEFAs levels. Human non-alcoholic fatty liver disease (NAFLD) is associated with NEFAs-induced hepatocyte apoptosis and insulin resistance [24]. Herdt [3] found that poor feedback control during NEFAs release from adipose tissue is an important cause of ketosis and fatty liver.

ROS can cause liver dysfunction by generating harmful substances and reducing bile production. The resulting imbalance in biological toxin and drug toxin metabolism in hepatocytes can lead to subclinical hepatitis, cirrhosis and liver cancer [25]. Schonfeld and Wojtczak [7] demonstrated that NEFAs can cause oxidative stress to cells through several mechanisms. In our study, NEFAs reduced the TAC and GSH content and the activity of GSH-Px, CAT and SOD in hepatocytes but simultaneously increased the MDA, GSSG and ROS content. NEFAs reduced the GSH/GSSG rates. These results demonstrate that NEFAs can induce oxidative stress in bovine hepatocytes. When NEFAs were added with glucose, which functions as an energy supply, oxidative stress and apoptosis in the hepatocytes were inhibited. This finding indicates that hepatocytes prefer glucose, which decreases the lipotoxicity of NEFAs. These results demonstrate the indispensable role of NEB in initiating damage. Our findings agree with previous studies on NEFAs.

As reported in a series of studies, NEFAs can act as signaling molecules that promote apoptosis by affecting the expression of apoptosis genes [26]. One potential mechanism involves the induction of apoptosis by NEFAs through the production of ROS. We showed via both observation of apoptotic phenomena and transcriptional and translational analysis of several apoptosis-related genes that high concentrations of NEFAs elevated the apoptotic rate of hepatocytes. GLU is the main energy supply molecule in hepatocytes, while NAC is an antioxidant that can reduce the production of ROS. We found that GLU and NAC protected hepatocytes from the apoptosis caused by NEFAs. These results suggest that NEFAs can induce oxidative stress-mediated hepatocyte apoptosis.

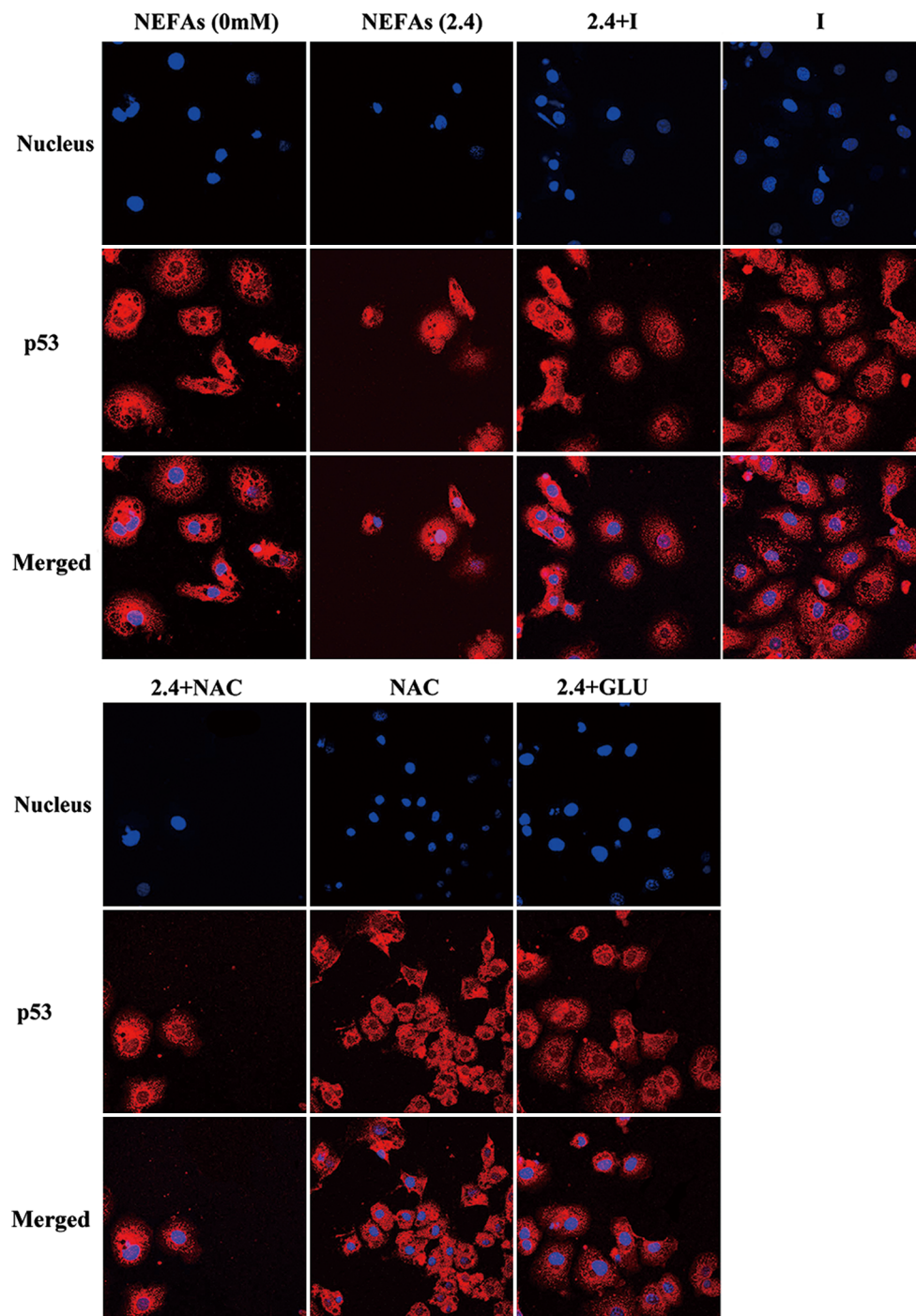


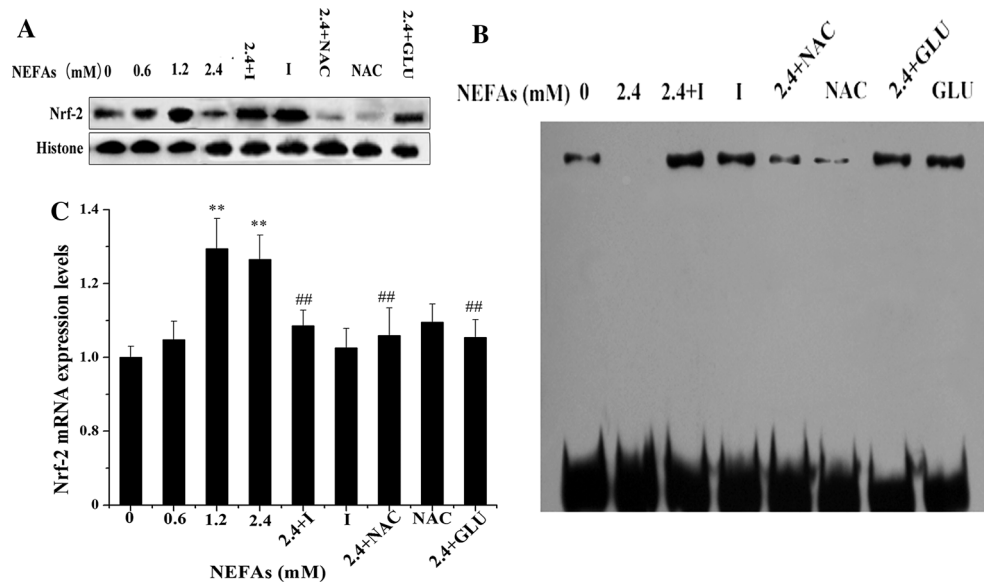
Fig. 6 p53 participates in apoptosis by p38-mediated nuclear localization after treatment with NEFAs. The hepatocytes were treated with NEFAs for 9 h. p53 cellular localization was analyzed by immunofluorescence assay under a laser confocal microscopy respectively ($\times 600$)

Dairy cows with ketosis are in a high-fat and low-glucose state. High NEFAs levels can cause oxidative stress, which can result in cell and organ damage, inflammation and apoptosis through the activation of several signaling pathways, including the MAPK signaling pathway. The low level of glucose in cows with ketosis cannot protect hepatocytes from oxidative stress damage and instead

worsens the condition. Recent studies have also found that increased NEFAs concentrations in human blood are linked to insulin resistance, inflammation, metabolic syndrome and matrix metalloproteinases and that NEFAs are a therapeutic target in these conditions [27].

p38 MAPK is an important signal transduction mediator that responds to a wide range of extracellular stresses,

Fig. 7 The effects of NEFAs on the expression and transcriptional activity of Nrf2 in bovine hepatocytes. The hepatocytes were treated with NEFAs for 9 h. **a** Nuclear Nrf2 protein levels detected by western blot. **b** mRNA expression levels of Nrf2 detected by RT-PCR. **c** The transcriptional activity of Nrf2 detected by EMSA. * $p < 0.05$; ** $p < 0.01$ versus the control group (NEFAs 0 mM). # $p < 0.05$, ## $p < 0.01$ versus the 2.4 mM NEFAs group



particularly oxidative stress. ROS play a critical role in cytokine-induced p38 α activation, and p38 MAPK functions as a sensor of oxidative stress [12]. The activation of p38 MAPK affects the regulation of downstream transcription factors, such as p53 and Nrf2, to control the expression of downstream pro-apoptotic and anti-apoptotic genes [13].

The results regarding the function of p38 in apoptosis vary [28]. The role of p38 in apoptosis is cell type- and stimulus-dependent. While p38 signaling has been shown to promote cell death in some cell lines, p38 enhances survival, cell growth and differentiation in other cell lines [29]. In the present study, the expression levels of p38 were elevated in a NEFAs dose-dependent manner. The activity of p38 α was also increased in NEFAs-treated hepatocytes. Both NAC and GLU decreased the elevated p38 mRNA expression and apoptotic levels caused by NEFAs in hepatocytes. When treated with the p38 inhibitor SB203580, the apoptotic level decreased in hepatocytes. SB203580 also inhibited the expression of some pro-apoptotic genes but increased the expression of anti-apoptotic genes. Overall, NEFAs caused hepatocyte apoptosis that was mediated by the activation of p38. p38 was involved in the response to oxidative stress caused by high concentrations of NEFAs in cows with ketosis or fatty liver. p38 could mediate hepatocyte death or preservation by regulating downstream transcription factors, including p53 and Nrf2 [14, 15].

p53 is a redox-sensitive transcription factor. The expression and activity of the p53 gene increase during oxidative stress. Many mechanisms regulate p53 post-translationally, such as phosphorylation, acetylation, ubiquitination and glycosylation. All of these mechanisms

constitute a complex control system that finely adjusts the cellular localization and function of p53 as well as DNA repair, cell senescence and apoptosis [30]. p53 can be activated by many kinases, including p38, which stabilizes p53 and promotes its accumulation in the nucleus. p53 can also act as a transcription factor to regulate the expression of its target genes (e.g., Bax, Bak, Puma, Noxa and Apaf1), which can affect DNA repair [31]. Here, we found that both the levels and activity of p53 were increased by NEFAs, and high concentrations of NEFAs promoted p53 mRNA expression and nuclear localization. Activated p53 increased the expression of the pro-apoptotic genes Bax, Caspase 3 and Caspase 9 and the activation of the pro-apoptotic proteins Caspase 3 and PARP. However, NEFAs decreased the expression of Bcl-2. SB203580, NAC and GLU countered the effects of NEFAs. These results demonstrate that p53 is a key transcription factor downstream of p38 in hepatocytes treated with NEFAs. We also showed that p53 was involved in the ROS/p38-mediated hepatocyte apoptosis caused by NEFAs.

Nrf2 is a double-edged sword because it inhibits chemical carcinogenesis but increases cancer cell survival and promotes drug resistance [32]. Nrf2 is a key regulatory factor that can protect cells from oxidative stress. Some studies have demonstrated that p38 is involved in regulating the phosphorylation of Nrf2. Some evidence suggests that p38 can promote the dissociation of Nrf2 from the Nrf2–Keap1 complex by phosphorylating Nrf2 directly or through intermediary kinases, thus permitting liberated Nrf2 accumulation in the nucleus and regulating the activation of some antioxidant target genes [33].

Other studies have described a different response of p38–Nrf2 to oxidative stress. Li et al. [34] found that in

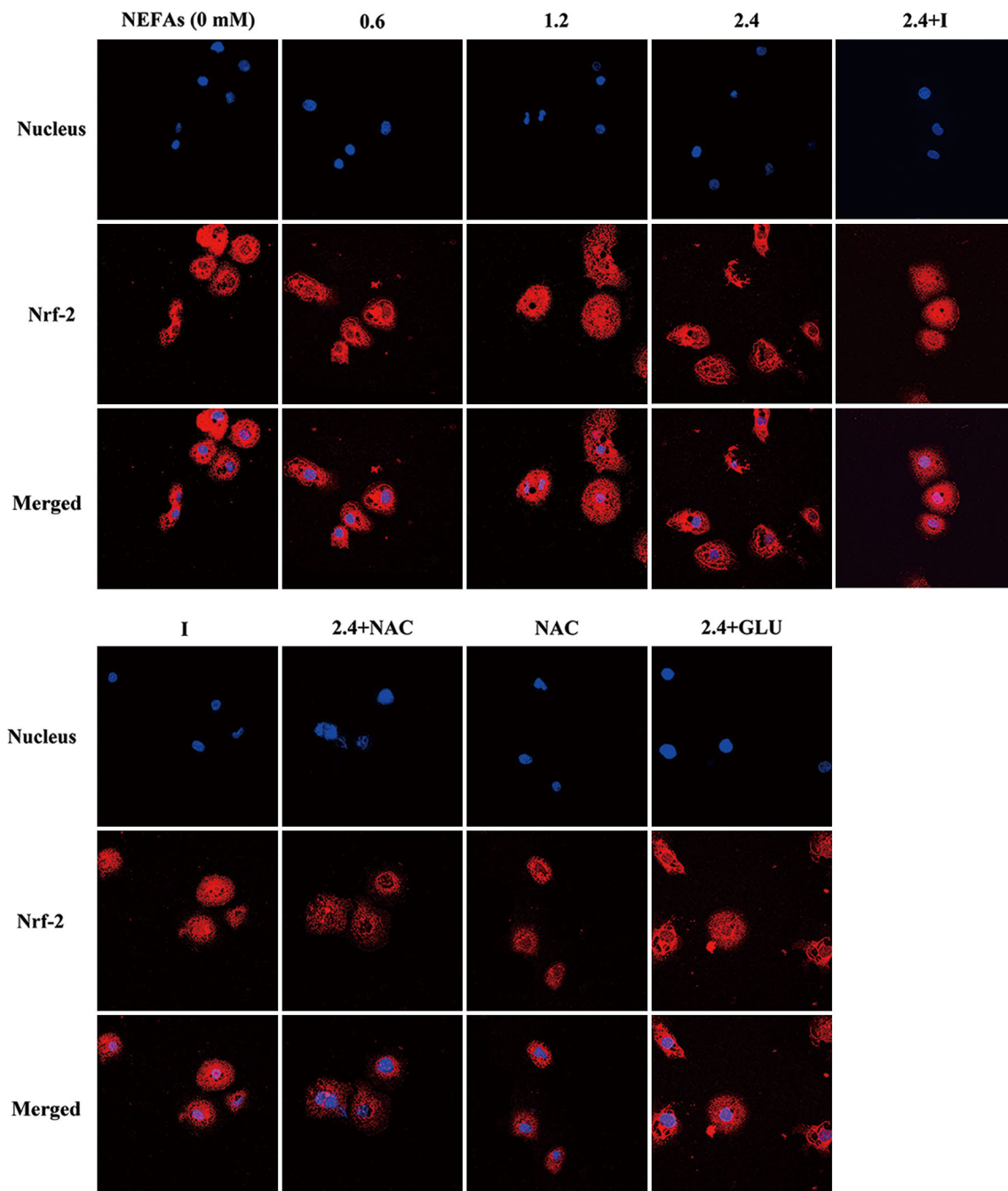


Fig. 8 Nrf2 participates in apoptosis by p38-mediated nuclear localization after treatment with NEFAs. The hepatocytes were treated with NEFAs for 9 h. Nrf2 cellular localization was analyzed by immunofluorescence assay under a laser confocal microscopy respectively ($\times 600$)

manganese-induced oxidative stress in PC12 cells, the key gene regulating oxidative stress, Nrf2, was activated by the ubiquitin–proteasome pathway and did not involve p38. In contrast, Shen et al. [35] have shown that activated Erk and the JNK pathway induce Nrf2 activation to prevent oxidative stress and that the p38 signaling pathway plays the opposite role. Our results showed that the mRNA expression levels of Nrf2 were elevated in NEFAs-treated

hepatocytes. Relative to the 2.4 mM NEFAs treatment group, SB203580, NAC, GLU weakened the effects of NEFAs on Nrf2. GLU increased the transcriptional activity of Nrf2. Interestingly, the western blot and immunofluorescence assays showed that the nuclear protein content and nuclear localization increased for NEFAs concentrations up to 2.4 mM but decreased dramatically in the 2.4 mM NEFAs treatment group compared with the control

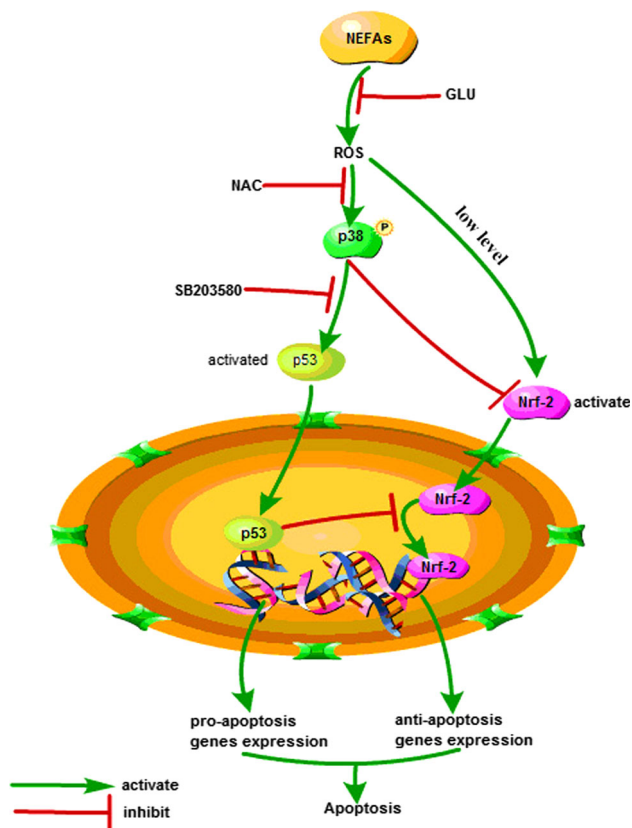


Fig. 9 Signal activation pathways in hepatocytes treated with NEFAs

group. High concentrations of NEFAs and NAC markedly decreased the transcriptional activity and nuclear localization of Nrf2 in hepatocytes. These results suggest that Nrf2 may protect cells from oxidative stress responses during the early stage or low-level oxidative stress caused by NEFAs. However, when the hepatocytes were treated with 2.4 mM NEFAs, the antioxidant effect of Nrf2 was inhibited, thereby inducing disequilibrium of apoptosis and anti-apoptosis. Relative to the 2.4 mM NEFAs group, the SB203580 + 2.4 mM NEFAs treatment group had higher nuclear protein content, transcriptional activity and localization levels of Nrf2, which suggests that SB203580 increased the nuclear accumulation of Nrf2. SB203580 inhibited the phosphorylation of p38 MAPK and then increased the nuclear accumulation of Nrf2. These findings suggest that the activation of p38 caused by NEFAs can inhibit the transcriptional activity and nuclear localization of Nrf2. Additional evidence indicates that Nrf2 and p53 are functional competitors for the same DNA promoter regions, and p53 suppresses the Nrf2-dependent transcription of antioxidant response genes [36]. In this study, NEFAs activated ROS-mediated p38 MAPK and increased the expression and transcriptional activity of p53, which may inhibit the transcriptional activity, nuclear protein content and nuclear localization of Nrf2 and weaken the

expression of anti-apoptotic genes. However, more evidence is required to accurately identify the molecular mechanism of the inhibition of Nrf2 by p53.

In summary, our results suggest that high levels of NEFAs induce oxidative damage and apoptosis in bovine hepatocytes. Importantly, we confirmed that the ROS–p38–p53/Nrf2 pathway is involved in the control of NEFAs-induced apoptosis (Fig. 9). These findings will promote new exploration into the prophylaxis and treatment of metabolic disorders induced by NEFAs in dairy cows with NEB.

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