

Original Paper

Ghrelin Attenuated Lipotoxicity via Autophagy Induction and Nuclear Factor- κ B Inhibition

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Key Words

Nonalcoholic fatty liver disease (NAFLD) • Free fatty acid (FFA) • High fat diet (HFD) • Autophagy • Ghrelin • Nuclear factor- κ B

Abstract

Background/Aims: Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. Autophagy is associated with NAFLD. Ghrelin is a gut hormone with various functions including energy metabolism and inflammation inhibition. We investigated the therapeutic effect of ghrelin on NAFLD and its association with autophagy. **Methods:** C57bl/6 mice were fed a high-fat diet for 8 weeks to induce a model of chronic NAFLD, with ghrelin (10 μ g/kg) administered subcutaneously twice weekly from weeks 6 to 8. LO2 cells were pretreated with ghrelin (10⁻⁸ M) before stimulation with free fatty acid (palmitic and oleic acids; 1 mM). Lipid droplets were identified by hematoxylin and eosin and Red O staining and quantified by triglyceride test kits. LC3I/II, an important biomarker protein of autophagy was detected by western blotting, real-time polymerase chain reaction, immunohistochemistry and immunofluorescence. Tumor necrosis factor (TNF)- α and interleukin (IL)-6 were detected by ELISA and immunohistochemistry. Nuclear factor (NF)- κ B p65 was detected by western blotting and immunofluorescence. AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) were detected by western blotting. **Results:** Ghrelin reduced the triglyceride content in high fat diet (HFD) group *in vivo* and free fatty acid (FFA) group *in vitro*. TNF- α and IL-6 were significantly reduced in the ghrelin-treated mice compared with the control group. Autophagy induction was accompanied with intracellular lipid reduction in ghrelin-treated mice. Ghrelin upregulated autophagy via AMPK/mTOR restoration and inhibited translocation of NF- κ B into the nucleus. **Conclusions:** The results indicate that ghrelin attenuates lipotoxicity by autophagy stimulation and NF- κ B inhibition.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide. The prevalence of NAFLD in adults in the US is currently estimated at $\leq 46\%$ [1], which is of major concern, with its progression from steatosis to steatohepatitis, cirrhosis, and even hepatocellular carcinoma. At present, there are no effective drug therapies for NAFLD. According to the classical “two hits” theory [2], both insulin resistance and oxidative stress contribute to initiation and progression of NAFLD. Increased lipid is not offset by mitochondrial oxidation or by increased secretion of lipoproteins [3]. As a result, oxidative stress occurs, causing hepatocyte injury with reactive oxygen species (ROS), which lead to cell inflammation and even apoptosis, called lipoapoptosis [4]. Moreover, a recent study showed there is a critical role for miRNAs in the pathogenesis of NAFLD [5].

On the way from fat to inflammation, nuclear factor (NF)- κ B signaling pathway activation is characterized as the most important mechanism, which functions as a proinflammatory “master switch” by upregulating transcription of a wide range of inflammatory mediators [6]. Lipid accumulation induced by high-fat diet (HFD) in the liver of mice leads to subacute hepatic inflammation via NF- κ B activation, with increased downstream cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β [7].

Autophagy is an intracellular process of self-digesting excess or defective organelles to maintain homeostasis, which has entered the research spotlight due to its wide involvement in different diseases. Autophagy mainly acts as a cell survival mechanism. However, excessive autophagy may also contribute to cell death called type II programmed cell death, suggesting that autophagy is a double-edged sword [8]. The new function of autophagy in regulating intracellular lipid stores is called macrolipophagy, which is regarded as liver-specific autophagy. The pharmacological inhibition of autophagy with 3-methyladenine (3-MA) and autophagy-related gene (Atg) knock down increase intracellular lipid storage [9]. Ablation of macrolipophagy leads to excessive hepatic lipid accumulation and development of NAFLD [10]. The latest research has discovered that caffeine stimulates hepatic lipid mechanism and increases hepatic fat clearance via the autophagy-lysosome pathway [11], which makes us more confident that autophagy is a new therapeutic target for NAFLD.

Ghrelin is a novel peptide that was first purified from the stomach, and is the endogenous ligand for growth hormone secretagogue receptor [12]. It is a brain-gut peptide existing in two forms, with the octanoyl form regarded as the active form. Circulating ghrelin is mostly produced in X/A-like cells of the stomach and small intestine. However, small amounts of ghrelin are also produced in other organs such as pancreas, lung, kidney and pituitary, and adipocyte tissue. Ghrelin is a multifunctional peptide showing roles in orexigen, energy regulation, and growth hormone release. Recently, ghrelin was demonstrated to improve inflammatory reactions by enhancing immune-cell proliferation and inhibiting secretion of proinflammatory cytokines [13]. Li et al. first demonstrated that ghrelin administration in rats attenuated NAFLD-induced hepatic injury, inflammation and apoptosis, partly through restoration of LKB1/AMPK and PI3K/Akt pathways [14], indicating that ghrelin is a new treatment for NAFLD.

However, the mechanisms of ghrelin-regulated lipid metabolism are not clear. Research in rodent models of NAFLD is limited. In some studies, ghrelin was reported to improve cardiac cell injury by inducing autophagy [15] or inhibiting excessive autophagy [16]. However, whether ghrelin is associated with autophagy in ameliorating NAFLD-induced liver injury has not been investigated. The study was designed to investigate whether ghrelin reduced hepatic triglyceride and inflammation injury in NAFLD, with or without participation of autophagy.

Materials and Methods

Reagents

Ghrelin was purchased from Prospec (Israel). HFD (D12492), composed of 20% protein, 60% fat, and 20% carbohydrate of the caloric intake; 5.24 kcal/g was from Shanghai QF Biosciences (Shanghai, China).

The following chemicals were all from Sigma-Aldrich (St Louis, MO, USA): bovine serum albumin (BSA), oleic acid (OA), palmitic acid (PA), 3-methyladenine (3-MA), and rapamycin. Antibodies against NF- κ B p65 were purchased from Abcam (Cambridge, MA, USA). The following antibodies were all purchased from Cell Signaling Technology (Danvers, MA, USA): LC3, Beclin 1, I κ B α , phosphorylated AMPK (p-AMPK) at Thr172, total AMPK, phosphorylated mTOR(p-mTOR), total mTOR and lamin-A.

Cell culture and treatment

Normal human liver cell line LO2 was purchased from the Chinese Academy of Science Committee Type Culture Collection Cell Bank. Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, South America) and 1% penicillin-streptomycin (Gibco, USA) in a humidified incubator at 37°C in 5% CO₂. OA and PA (20 mM solution) were dissolved in 5% BSA which had been previously heated at 37°C in a water bath for several hours. LO2 cells were exposed to 1 mM free fatty acid (FFA) mixture (OA: PA, 2:1) for 24 h to induce steatosis. Cells were pretreated with ghrelin at concentration of 10 nM for 1 h before stimulation with FFA. Cells were divided into four groups: 1. Normal control group (NC): treated with PBS only as vehicle; 2. NC+ ghrelin group: treated with ghrelin diluted in PBS at a concentration of 10⁻⁸M; 3. FFA group: treated with 1 mM FFA mixture for 24 h; and 4. FFA+ ghrelin group: treated with ghrelin and FFA mixture described above.

Animals and treatment

C57BL/6 mice (8 weeks old, 23 ± 2 g) were purchased from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China). The mice were housed in a clean room maintained at 24 ± 2°C with an alternating 12-h light and dark cycle, with free access to food and water. All animal experiments were approved by the Animal Care and Use Committee of Shanghai Fudan University. Mice were randomly divided into four groups with 7 mice each group. 1. Saline group: mice were fed with normal rat diet with saline injected intraperitoneally daily from week 6 to 8. 2. Saline+ ghrelin group: mice were fed with normal rat diet for 8 weeks, with ghrelin injected intraperitoneally daily from week 6 to 8 at a dose of 10 µg/kg. 3. HFD group: mice were fed with HFD for 8 weeks. 4. HFD+ ghrelin group: mice were fed with HFD for 8 weeks with ghrelin injected intraperitoneally twice weekly from week 6 to 8 at a dose of 10 µg/kg. All mice were sacrificed at 8th week. Liver samples and blood were acquired for histological and molecular analysis.

Biochemical assays

The serum levels of ALT and AST were measured with microplate test kits (Nanjing Jiancheng Bioengineering Institute, China). Serum proinflammatory cytokines TNF- α and IL-6 were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA). Triglyceride levels in LO2 cells were measured with a microplate test kit from Nanjing Jiancheng Bioengineering Institute.

Histopathology

The middle portion of the left lobe of the liver of each mouse was excised and sectioned and then perfused in 4% paraformaldehyde for at least 24 h. After fixation, the tissues were embedded in paraffin, and 5-µm thick sections were stained with hematoxylin and eosin (H&E) to observe the tissue damage by light microscopy.

Immunohistochemistry

The liver sections (3 µm) were dewaxed and rehydrated regularly and treated with 3% H₂O₂. The sections were pretreated with a microwave antigen retrieval technique. The nonspecific sites were blocked with 10% goat serum for 30 min at room temperature. The liver slices were incubated overnight with antibody directed against Beclin-1 (1:500) and LC3II (1:500). On the second day, after incubation with secondary antibody, an antibody binding assay was performed using a diaminobenzidine kit. Three different fields of vision were randomly selected in one slide, and the ratios of positive areas to total areas were acquired with Image-Pro Plus 6.0. We calculated the average of these three ratios. The above method was applied in all groups.

Western blotting

Liver tissues and LO2 cells were collected and lysed with RIPA buffer and protease inhibitor. The protein concentrations were measured with the BCA method. Equivalent amounts of total protein were separated by SDS-PAGE and transferred to PVDF membranes. Nonspecific binding was blocked with 5%

nonfat milk (dissolved in PBS) for 1 h and then incubated overnight at 4°C with antibodies directed against LC3II (1:500), Beclin-1 (1:1000), NF-κB p65(1:500), IκB (1:500), pAMPK (1:500), total AMPK (1:500), β-actin (1:1000), p-mTOR(1:500), mTOR(1:500) and lamin-A (1:500). All membranes were washed with PBS + 1% Tween (PBST) and incubated with a secondary antibody (1:1000) dissolved in PBST, for 1 h at 37°C. Finally, the membranes were washed three times with PBST for 10 min and the proteins were detected with the Odyssey two-color infrared laser imaging system (detected with fluorescence).

Oil Red O staining

LO2 cells were cultivated in six-well plates, processed with the relevant treatment, rinsed three times with PBS, fixed in 4% paraformaldehyde for 30 min, stained for 15 min at room temperature in freshly diluted Oil Red O, and rinsed twice with PBS. Finally, intracellular lipid droplets were observed with a light microscope.

Immunofluorescence staining

Immunofluorescence staining was performed according to standard protocols. The LO2 cells were washed three times with PBS solution for 1 min. The cells were fixed with 4% paraformaldehyde for 30 min and washed three times with PBS for 5 min. Nonspecific antigen-binding sites were blocked with 5% BSA and cells were incubated overnight with anti-LC3II antibody (1: 50) at 4°C. On the second day, cells were washed three times with PBS for 5 min and incubated with anti-rabbit antibody for 30 min. 2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI) (Life Technologies, USA) was used to stain cell nuclei. Tandem GFP-tagged LC3 virus (Invitrogen, Carlsbad, CA, USA) was used to infect cells for 24 h. All images were acquired using ZEN software (Carl Zeiss AG, Germany).

RNA isolation and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The liver tissue and LO2 cells were detected and analyzed with qRT-PCR. Total RNA was extracted from frozen liver tissues or cells with TRIzol reagent (Tiangen Biotech, China). SYBR Green Quantitative RT-PCR was performed with the 7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA) to determine expression of the target genes, according to the instructions for SYBR Premix EX Taq (TaKaRa Biotechnology, China). The primer sequences are shown in Table 1.

Electron microscopy

Mice and LO2 cells were treated as described above, and fresh liver tissue was perfused with 2 mL 2.5% glutaraldehyde in PBS. LO2 cells were collected from six-well plates and perfused with glutaraldehyde as described above. The tissue and cells were sectioned and examined under a transmission electron microscope (Tecnai) at 160 kV. Electron micrographs were obtained using Electron Microscopy Film 4489 (Kodak, ESTAR thick base) and printed onto photographic paper.

Statistical analysis

All results are expressed as means ± SD. Comparisons between two groups were made with Student's *t* test. Statistical differences in multiple groups were determined by multiple comparisons with analysis of variance, followed by Tukey's post hoc test. All statistical analyses were performed with Graphpad Prism version 6.0 software. *P* < 0.05 was considered statistically significant.

Table 1. Nucleotide sequences of primers used for PCR

Gene		Primer sequence (5'-3')
LC3	Forward	TGCTGTCCCGAATGTCTCCTG
	Reverse	GCTAACCAAGCCTTCTTCCTCC
Beclin-1	Forward	AGATGCCTCCCCGATCAGAG
	Reverse	CTTACCACAGCCCAGGCGAA
β-Actin	Forward	GGCTGTATTCCCCTCCATCG
	Reverse	CCAGTTGGTAACAATGCCATGT

Results

Ghrelin administration reduced lipid accumulation

Oil Red O staining of cells showed ghrelin pretreatment before FFA stimulation significantly reduced intracellular lipid accumulation, which was quantified by triglyceride test kit (Fig. 1A and 1B). The results were confirmed using H&E staining (Fig. 1D) and Oil Red O staining (Fig. 1E) of mice liver tissues. The liver was photographed and liver weight/body weight ratio was calculated. Actually we used two doses of ghrelin to treat HFD mice (10 ng/kg and 10 µg/kg) in the preliminary experiment and found high dose of ghrelin group showed better effect. Therefore, we used 10 µg/kg ghrelin in the following experiment. The HFD mice had hepatomegaly due to swollen hepatocytes and more shallow hepatic color visually, while the liver tissue in the ghrelin co-treatment group was nearly normal size and fresh red visually especially the high dose of ghrelin group. HFD led to a higher liver/body weight ratio compared with that in the saline group, which was significantly reduced by 10 µg/kg ghrelin treatment, as well as the indicators of liver triglyceride level and serum cholesterol level (Fig. 1C).

Ghrelin administration attenuated hepatic inflammation induced by lipotoxicity

As indicators of liver injury, elevated serum levels of ALT and AST were observed in HFD group, which were significantly reduced by co-treatment with ghrelin (Fig. 2A). Serum levels of pro-inflammatory cytokines (TNF-α and IL-6) were significantly upregulated in HFD mice and down-regulated in the ghrelin+ HFD group (Fig. 2B). Immunohistochemical staining showed ghrelin significantly reduced the expression of TNF-α and IL-6 compared with HFD group (Fig. 2C and 2D). There were no significant differences of hepatic enzymes or pro-inflammatory cytokines between the supernatant of cultured LO2 cells from the NC, FFA and FFA+ ghrelin groups (data not shown).

Ghrelin-induced lipid clearance was associated with a concomitant increase in autophagy

Five methods were used to examine the relationship between lipid clearance and autophagy including western blotting, qRT-PCR, immunohistochemistry, immunofluorescence and electron microscopy. Increased protein levels of LC3 and Beclin-1 were observed in the HFD or FFA group, which were further stimulated by ghrelin co-treatment *in vivo* and *in vitro* (Fig. 3 and 4). We investigated mRNA transcription level of LC3 and Beclin-1 and found similar results. Protein levels of LC3II and Beclin-1 were also measured by immunohistochemistry (Fig. 4B). Immunofluorescence of LC3II showed that autophagic flux was increased in HFD group and FFA group, while ghrelin treatment further activate it both *in vitro* and *in vivo* (Fig. 3B and Fig. 4C). Similarly, adenovirus carrying GFP-LC3 detected increased expression of LC3II in ghrelin co-treated group (green dots in merged images shown in Fig. 3C). Electron microscopy showed increased autophagosomes in the ghrelin co-treated group *in vivo* and *in vitro* (Fig. 3D and Fig. 4D).

Autophagy inhibition reversed reduction of intracellular lipid droplets

To investigate whether the ghrelin-induced reduction in intracellular triglyceride was reversed by autophagy inhibition, 10 mM 3-MA (autophagy inhibitor) and 1 µM rapamycin (autophagy enhancer) were used to treat LO2 cells. Reduced level of LC3II was detected in the 3-MA+ ghrelin+ FFA group compared with the FFA+ ghrelin group. On the contrary, increased level of LC3II was observed in the rapamycin+ ghrelin+ FFA group, indicating that the ghrelin-induced increase in autophagy was inhibited by 3-MA and augmented by rapamycin stimulation. The intracellular lipid level in the 3-MA+ghrelin+ FFA group was higher than that in the ghrelin+ FFA group, while rapamycin treatment significantly reduced intracellular lipid accumulation of steatotic LO2 cells (Fig. 3E).

Ghrelin promoted autophagy partly via restoration of the AMPK/mTOR signaling pathway

HFD or FFA dramatically reduced phosphorylation of AMPK (pAMPK) while ghrelin restored it without affecting the level of total AMPK. As shown in Fig. 5, mTOR, an AMPK

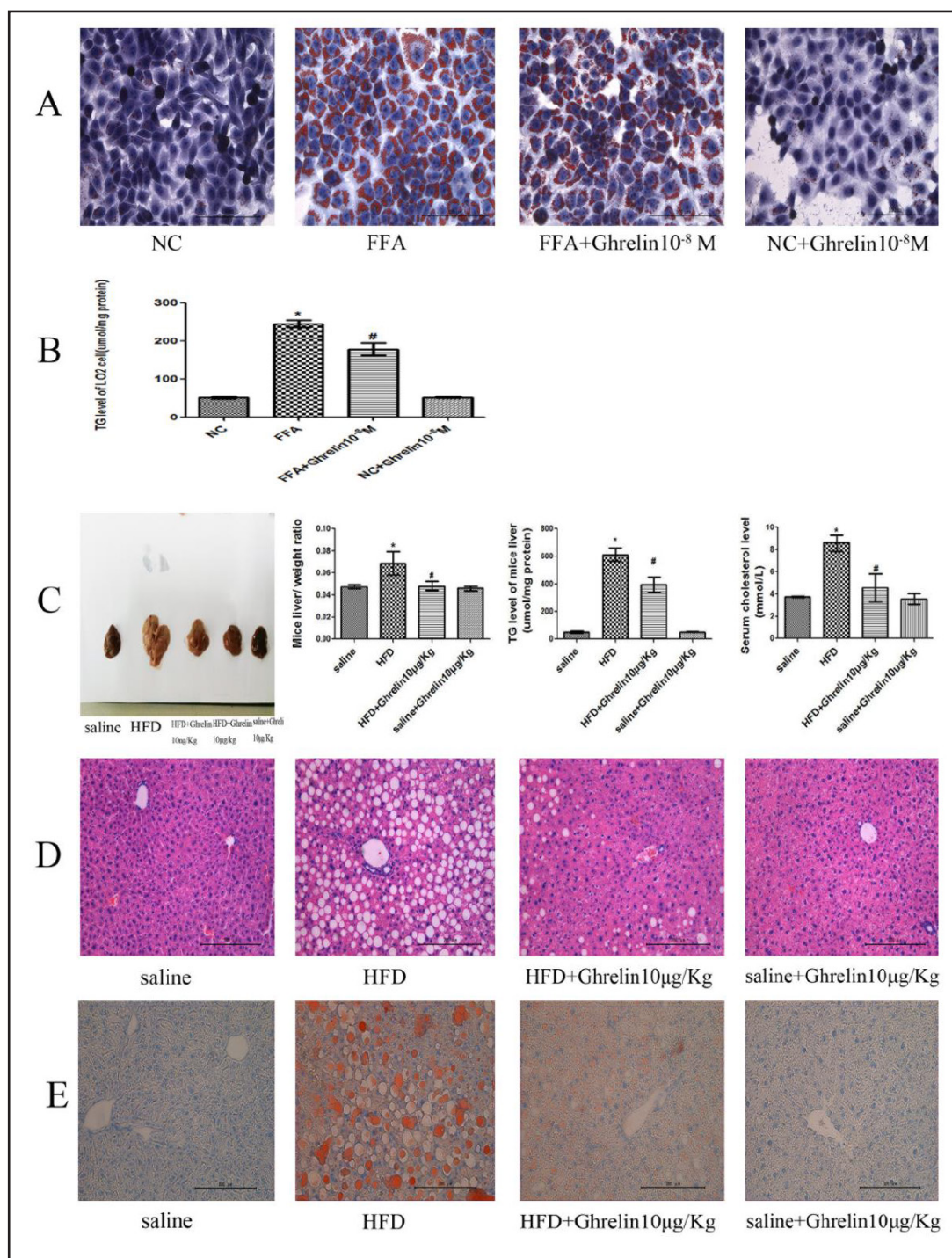


Fig. 1. Ghrelin reduced lipid accumulation *in vitro* and *in vivo*. (A) Representative images of LO2 cells stained with Oil Red O, which were exposed to 1 mM FFA mixture (OA and PA; 2:1) and ghrelin (10^{-8} M) for 24 h, compared to FFA-treated cells, NC group and NC+ ghrelin group. (B) Intracellular triglyceride levels were quantified in the four groups. * $P < 0.05$ for FFA group versus NC group; # $P < 0.05$ for FFA+ ghrelin group versus FFA group. (C) Liver from mice groups was photographed: saline group; HFD group; HFD+ ghrelin group (low-dose ghrelin 10 ng/kg, high-dose ghrelin 10 μ g/kg). Mouse liver/body weight ratio, liver triglyceride content, and serum cholesterol level were detected. * $P < 0.05$ for HFD group versus saline group; # $P < 0.05$ for HFD+ ghrelin group versus HFD group. (D) H&E staining of mouse liver sections from the four groups described above. (E) Representative images of liver sections stained with Oil Red O from the above groups.

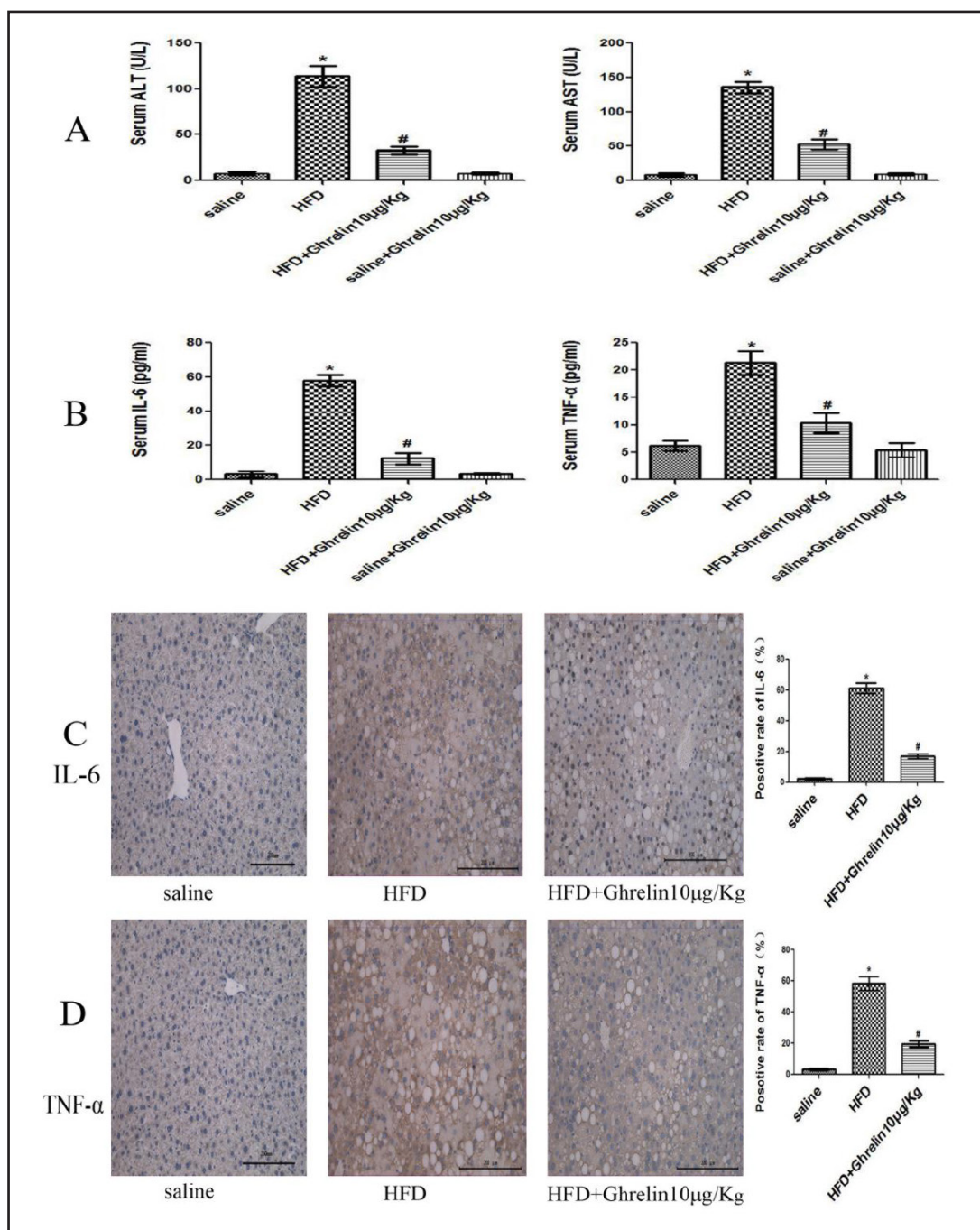


Fig. 2. Ghrelin administration attenuated hepatic inflammation induced by lipotoxicity. (A) Serum level of serum ALT and AST level were detected by ELISA in mice groups with or without ghrelin co-treatment. (B) Serum level of pro-inflammatory cytokines IL-6 and TNF- α were detected by ELISA in mice groups with or without ghrelin co-treatment. (C and D) Immunohistochemistry staining (200 \times) showed protein level of TNF- α and IL-6 in liver tissue of mice after NAFLD induction, with or without ghrelin co-treatment. * P <0.05 for HFD group versus saline group; # P <0.05 for HFD+ ghrelin group versus HFD group.

downstream and its phosphorylation form (p-mTOR) were detected in mice groups. Ghrelin treatment inhibited mTOR phosphorylation *in vivo* and *in vitro*. We demonstrated that ghrelin attenuated lipotoxicity by enhancing autophagy partly via restoration of the AMPK/mTOR signaling pathway.

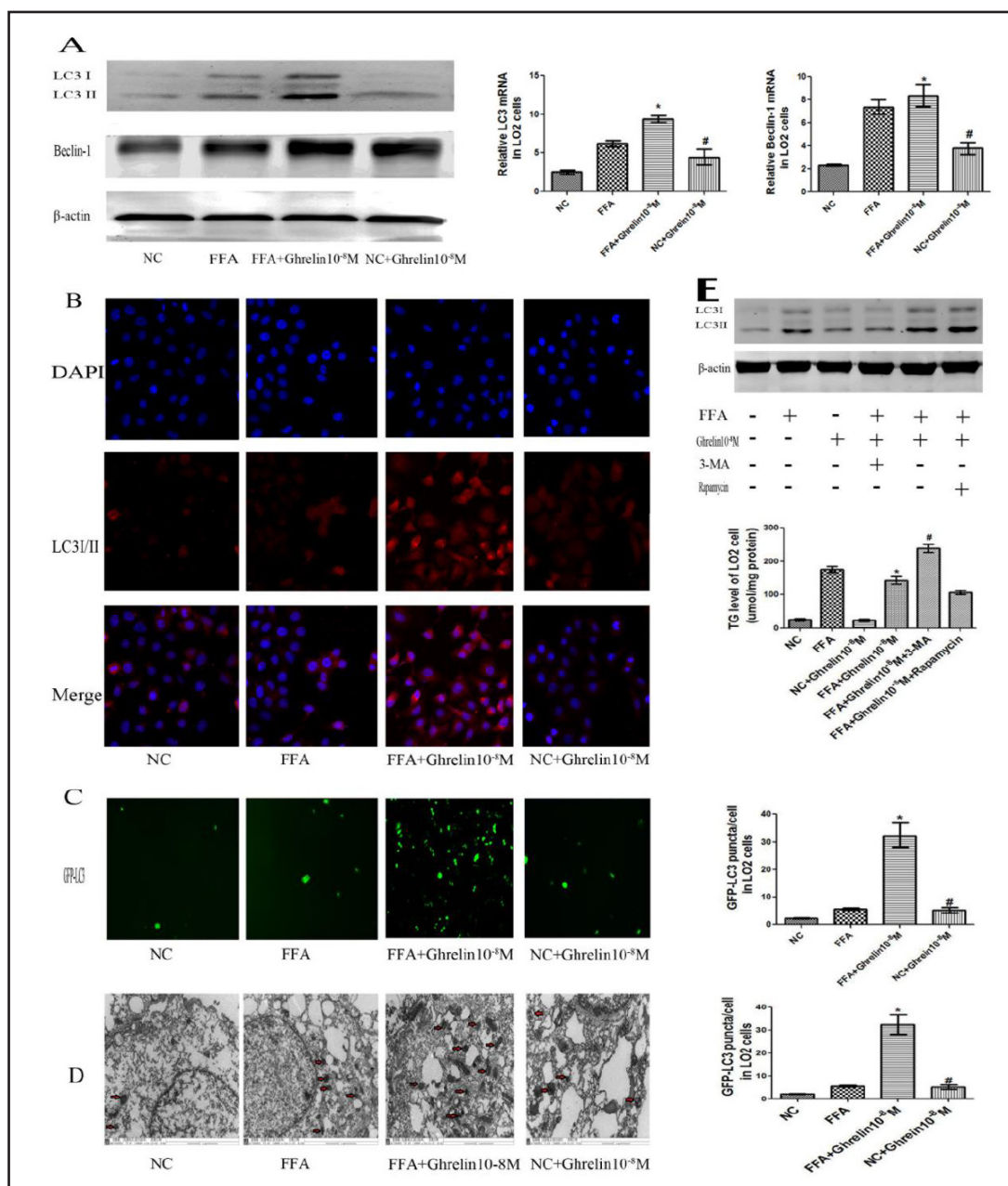


Fig. 3. Ghrelin-induced lipid clearance is associated with a concomitant increase in autophagy in LO2 cells. (A) Protein level of LC3I/II and Beclin-1 were detected by western blotting and mRNA expression of LC3I/II and Beclin-1 was detected by real-time qRT-PCR. * $P < 0.05$ for FFA+ ghrelin group versus FFA group; # $P < 0.05$ for NC+ ghrelin group versus NC group. (B) LC3 immunostaining showed increased endogenous LC3-II level in LO2 cells, which were pretreated with ghrelin (10⁻⁸ M) before stimulation with FFA, compared with cells treated with FFA alone, NC group and NC+ ghrelin group. (C) Adenovirus carrying GFP-LC3 detected the expression of LC3II *in vitro*. The average number of autophagosomes/cell \pm SD counted from fluorescence microscopy images of LO2 cells expressing GFP-LC3 (green dots in merged images shown in Fig. 3C, Original magnifications: $\times 400$). * $P < 0.05$ for FFA+ ghrelin group versus FFA group; # $P < 0.05$ for NC+ ghrelin group versus NC group. (D) Electron microscopy showed the ultrastructure and autophagosomes (indicated by \rightarrow). (Original magnifications: $\times 10000$). (E) Protein level of LC3I/II was detected by western blotting in the groups treated with or without autophagy inhibitor 3-MA or stimulator rapamycin in LO2 cells. Intracellular triglyceride levels were detected. * $P < 0.05$ for FFA+ ghrelin group versus FFA group; # $P < 0.05$ for FFA + ghrelin+3-MA group versus FFA+ ghrelin group.

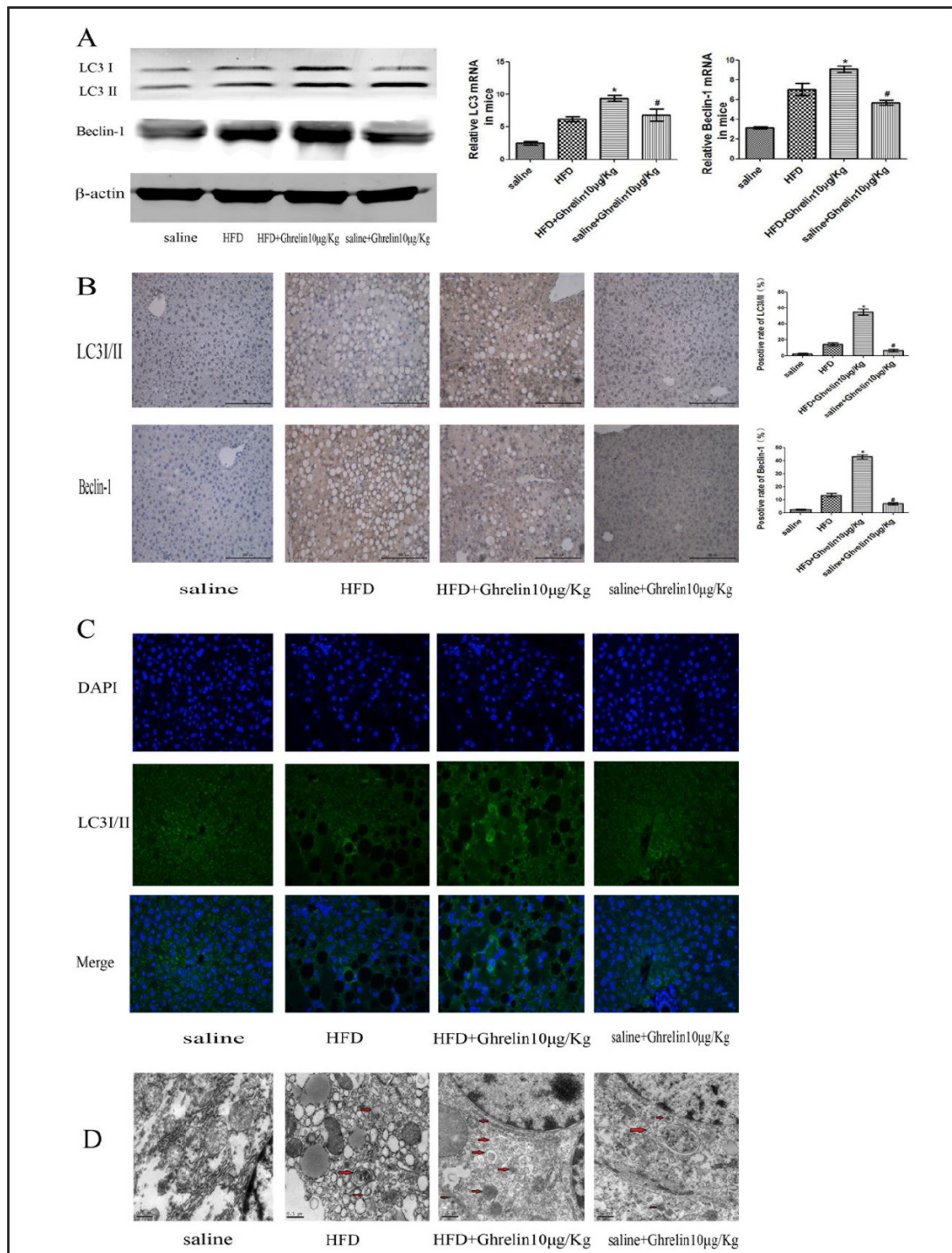


Fig. 4. Ghrelin-induced lipid clearance was associated with a concomitant increase in autophagy in mice. (A) Protein level of LC3I/II and Beclin-1 in mice was detected by western blotting and mRNA expression of LC3I/II and Beclin-1 was detected by real-time qRT-PCR. * $P < 0.05$ for HFD+ ghrelin group versus HFD group; # $P < 0.05$ for saline+ ghrelin group versus saline group. (B) Immunohistochemistry staining (200 \times) showed level of LC3I/II and Beclin-1 in liver tissue. * $P < 0.05$ for HFD+ ghrelin group versus HFD group; # $P < 0.05$ for saline+ ghrelin group versus saline group. (C) LC3 immunostaining of mice liver tissues showed increased endogenous LC3-II level in HFD+ ghrelin mice group, compared with HFD group, normal group and saline+ ghrelin group. (D) Electron microscopy showed ultrastructure and autophagosomes (indicated by \rightarrow). (Original magnifications: $\times 20000$).

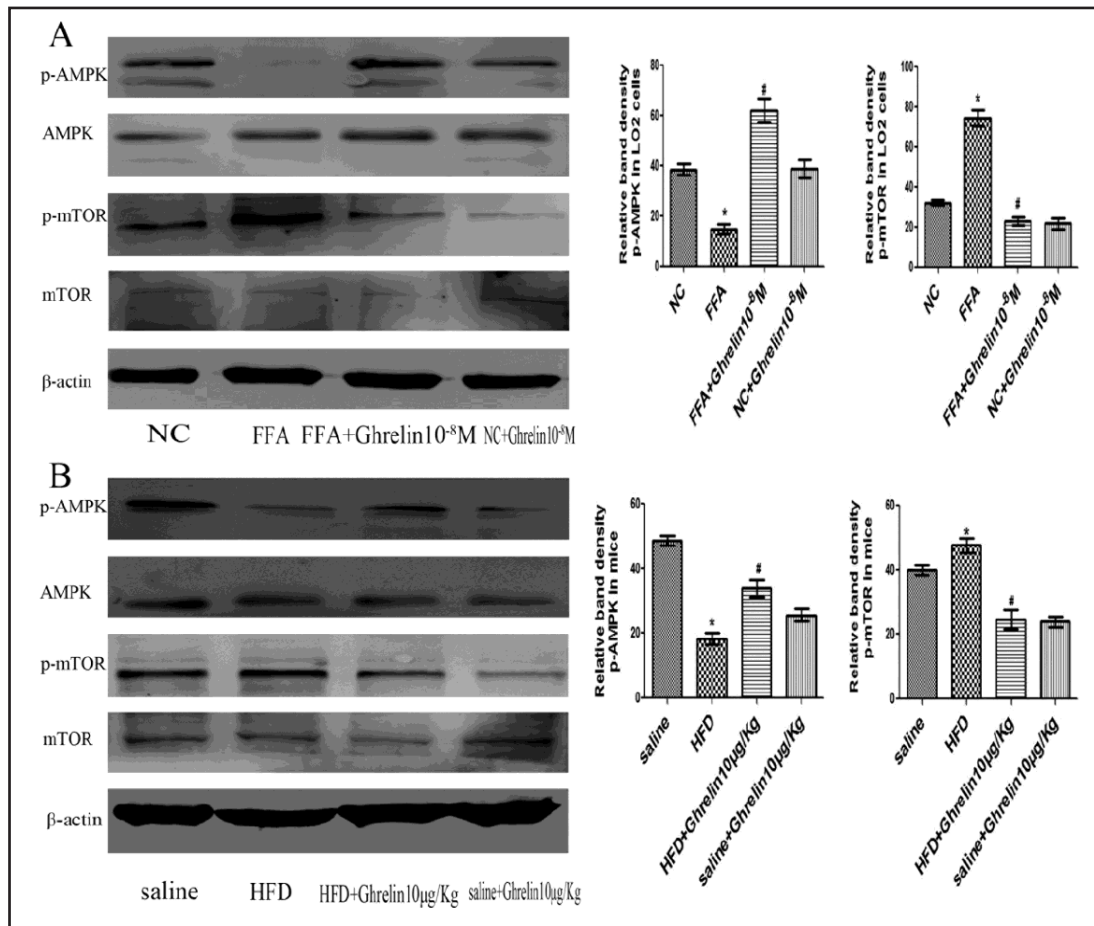


Fig. 5. Ghrelin promoted autophagy partly via restoration of AMPK/mTOR signaling pathway both *in vitro* and *in vivo*. (A and B) Western blots and quantitative evaluation of levels of p-AMPK, AMPK, p- mTOR and mTOR in LO2 cells and mice. * $P < 0.05$ for FFA group versus NC group in cells, HFD group versus saline group in mice; # $P < 0.05$ versus FFA+ ghrelin group versus FFA group in cells, HFD+ ghrelin group versus HFD group in mice.

Ghrelin attenuated inflammatory injury by NF-κB inhibition and autophagy enhancement

To investigate the changes in NF-κB nuclear translocation, which is known as the activated condition, we extracted the cytoplasmic and nuclear proteins from each group and detected the protein level of NF-κB. *In vitro*, the FFA group had a low level of NF-κB in the cytoplasm but a high level in the nucleus. On the contrary, the FFA+ ghrelin group had a higher level of NF-κB in the cytoplasm and a lower level in the nucleus. Protein levels of IκBα were reduced in the FFA group compared with the NC group, while ghrelin treatment ameliorated the decrease in IκBα protein compared with the FFA group (Fig. 6A). Similar results were obtained in mice experiment (Fig. 6B). Furthermore, immunofluorescence showed that FFA promoted NF-κB translocation in LO2 cells while ghrelin pretreatment reverse this process (Fig. 6C).

Discussion

NAFLD is being recognized increasingly as a major health burden [17]. It starts as a silent liver disease, but may progress into inflammation, fibrosis, and ultimately hepatoma. More importantly, NAFLD is closely associated with risk factors of coronary artery disease

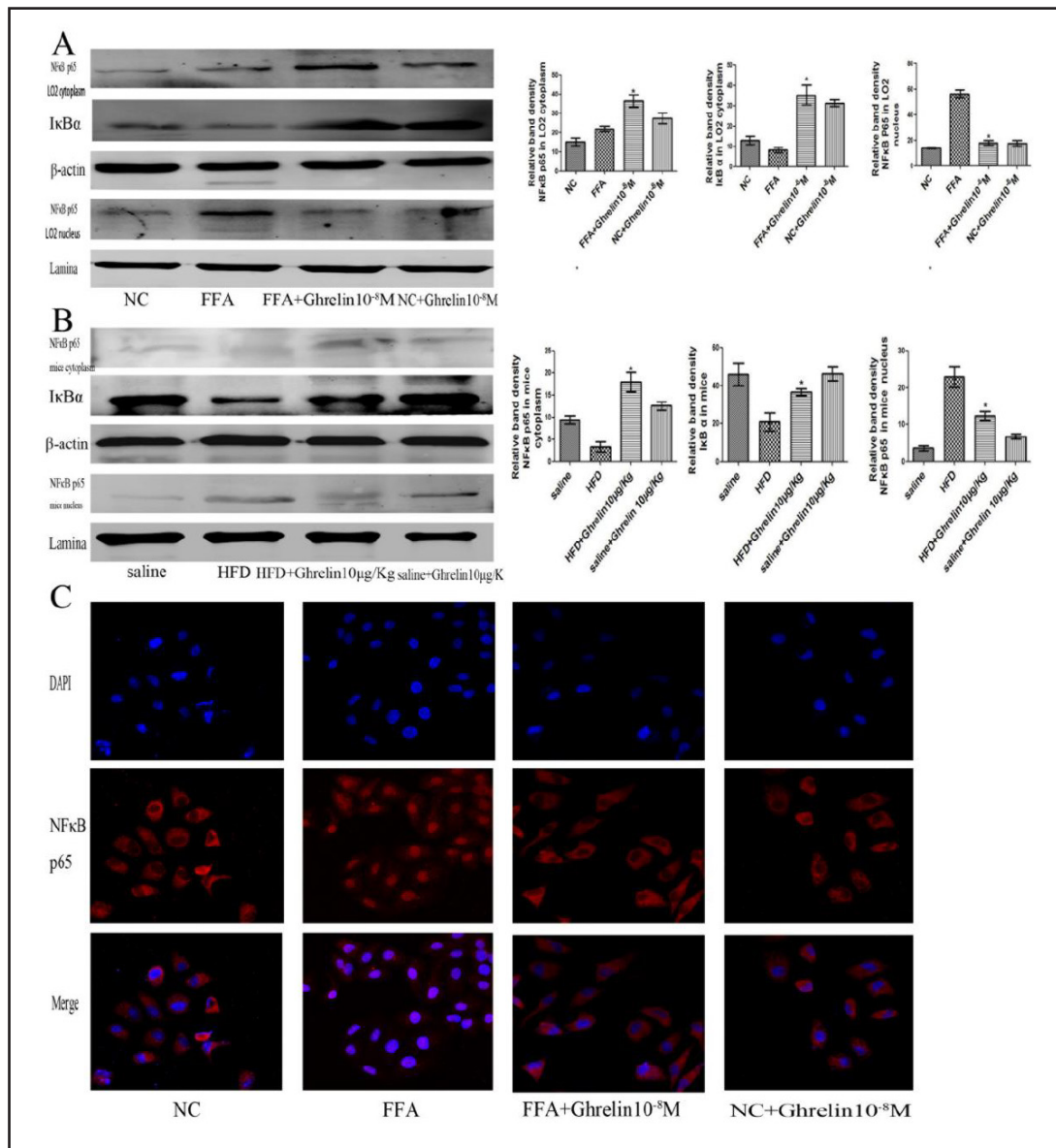


Fig. 6. Ghrelin administration attenuated inflammatory injury partly by NF-κB p65 inhibition both *in vitro* and *in vivo*. (A and B) Western blots and quantitative evaluation of level of IκBα and NF-κB p65 (cytoplasm and nucleus, respectively) *in vivo* and *in vitro*. *P < 0.05 for FFA+ ghrelin group versus FFA group in cells; HFD+ ghrelin group versus HFD group in mice. (C) Different distribution of NF-κB p65 in LO2 cells, with or without ghrelin pretreatment before FFA stimulation, was evaluated by immunofluorescence.

[18]. However, effective methods to prevent and attenuate NAFLD are urgently needed.

In this study, we presented a novel drug ghrelin, which could play a role in NAFLD therapy. Ghrelin is a newly discovered peptide with various functions, including regulating energy metabolism, promoting cell proliferation [19]. Recent studies have demonstrated that ghrelin attenuates inflammatory injury [13, 15, 16, 20, 21]. Okamatsu et al. have shown that serum ghrelin level is significantly elevated with liver function index in overweight children [22]. Likewise, another study has reported that high levels of ghrelin are associated with low risk of developing fatty liver [23]. The most recent study has confirmed our belief that ghrelin could have a therapeutic effect in NAFLD [20]. However, the therapeutic mechanism of ghrelin in NAFLD remains largely unknown, especially the way to reduce lipid

accumulation. Furthermore, the therapeutic effect of ghrelin has not been demonstrated *in vitro*. Our research is believed to be the first to demonstrate that ghrelin reduces lipid accumulation by enhancing autophagy, partly via restoration of the AMPK/mTOR signaling pathway.

We showed that ghrelin decreased intracellular lipids, with a concomitant increase in autophagy *in vitro* and *in vivo*. The dose at which we detected induction of autophagy *in vitro* was 10^{-8} M, as described previously [15]. The doses used in previous studies have been inconsistent. Therefore, we pretreated LO2 cells with a concentration gradient from 10^{-9} to 10^{-6} M before stimulation with FFA mixture in the preliminary experiment. LC3II protein level and intracellular triglyceride level were detected. However, we found that cells treated with 10^{-8} M ghrelin had the highest level of LC3II and lowest level of lipid (data not shown). We speculate that the effect of lipid reduction and autophagy was related to but not entirely concentration dependent. Whether ghrelin induces or inhibits autophagy is controversial. Wang et al. have shown that ghrelin inhibits doxorubicin cardiotoxicity by inhibiting excessive autophagy [24], which was contradicted with our research. We consider that the opposite effect of autophagy may be attributed to cell type, intervening measure and ghrelin concentration. Similarly, two doses of ghrelin (10 ng/kg and 10 μ g/kg) were used in HFD mice, and 10 μ g/kg showed a better therapeutic effect.

We observed a mild increase of LC3 protein level in HFD groups *in vivo* and FFA group *in vitro*, while ghrelin co-treatment significantly increased it. Short-term feeding with HFD in mice and FFA treatment *in vitro* promote autophagy as a self-protective mechanism to digest lipid partly via the mTOR complex, whereas long-term HFD inhibits autophagy by inhibition of the constitutive nuclear form of forkhead box O1, leading to acceleration of lipid accumulation [25]. Autophagy reduces lipids in NAFLD [9, 11] and in alcoholic fatty liver [26]. Triglyceride and cholesterol are taken up by autophagosomes and subsequently delivered to lysosomes for degradation into FFA, in a process known as lipophagy. Impaired lipophagy leads to excessive lipid accumulation [27]. Autophagy induction was accompanied with decreased intracellular lipid accumulation in our study. The ghrelin co-treated group had a significantly lower liver/body weight ratio and lipid accumulation *in vivo*. Furthermore, an autophagy inhibitor and stimulator (3-MA and rapamycin, respectively) were used to demonstrate that autophagy is essential for reduction of intracellular fat accumulation. As expected, 3-MA inhibited autophagy and increased triglyceride level. On the contrary, rapamycin lead to autophagy induction and obvious reduction of triglyceride level.

The AMPK signaling pathway plays a key role in the regulation of energy control. In starvation, AMPK is activated in response to an increase in AMP/ATP ratio, which requires phosphorylation of threonine 172 by an upstream kinase. AMPK is involved in the regulation of autophagy by inhibiting mTOR phosphorylation [28]. AMPK is inhibited by endoplasmic reticulum stress [29], oxidative stress and fat accumulation in NAFLD [14]. Furthermore, the effect of AMPK activation is stimulation of hepatic fatty acid oxidation and ketogenesis, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis [30], exerting an therapeutic effect on NAFLD. Ghrelin increases food intake and activates the AMPK signaling pathway [14, 20, 31]. Consistently, our study found that phosphorylation level of AMPK was reduced in the NAFLD group but restored by ghrelin without disturbing total AMPK. Meanwhile, p-mTOR was inhibited in the ghrelin co-treated group. Therefore, we speculate that ghrelin, an important energy regulator, stimulates appetite and activates AMPK, leading to the inhibition of p-mTOR and autophagy induction. NF- κ B signaling pathway is a classic pathway in inflammatory reaction which is well established on the way from fat to inflammation [6]. Lipotoxicity will promote the degradation of I κ B α and I κ B β , resulting in the translocation of NF- κ B into nucleus and subsequently the secretion of inflammatory cytokines. The anti-inflammatory effects of ghrelin are partly attributed to its NF- κ B inhibition. We showed that the I κ B α levels in the ghrelin co-treated group were markedly higher than those in the HFD *in vivo* or FFA group *in vitro*. While NF- κ B p65 was decreased in the HFD or FFA group but reversed in the cytoplasm in the ghrelin co-treated group. This demonstrates that ghrelin blocks I κ B α degradation and prevents translocation of NF- κ B

into the nucleus. Autophagy induction may be another way to explain the anti-inflammatory effect of ghrelin on lipotoxicity. Appropriate autophagy is regarded as a cellular survival mechanism by degrading excessive inflammasomes and attenuating oxidative and ER stress.

Autophagy activates hepatic stellate cells [32], which leads to liver fibrosis. We have to take into account that while ghrelin may attenuate NAFLD-induced liver injury and reduce lipid accumulation in the short term, its long-term effect in fibrogenic cells may worsen chronic liver disease. However, another study demonstrated that ghrelin ameliorated hepatic fibrosis in rodents by attenuating hepatocyte injury [33]. Whether autophagy is involved in this process needs further study. We recognize that there were some limitations to our study. We did not detect the oxidation reaction products of cells or liver tissues. Lipid dye used in the immunofluorescence study may help to show better the relationship between autophagy and lipid droplets. More strategies of autophagy inhibition such as gene knockout may be more compelling.

In conclusion, our research first demonstrates that ghrelin attenuated NAFLD-induced liver injury by autophagy induction via restoration of the AMPK/mTOR signaling pathway and NF- κ B inhibition. Energy regulators such as ghrelin offer further insight into developing drugs for the prevention and treatment of NAFLD.

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Disclosure Statement

The authors declare no conflict of interests.

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