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Original article

The preventive effect of liraglutide on the lipotoxic liver injury via increasing autophagy

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ABSTRACT

Introduction and objectives: The incidence of non-alcoholic fatty liver disease (NAFLD) is increasing. Previous studies indicated that Liraglutide, glucagon-like peptide-1 analogue, could regulate glucose homeostasis as a valuable treatment for Type 2 Diabetes. However, the precise effect of Liraglutide on NAFLD model in rats and the mechanism remains unknown. In this study, we investigated the molecular mechanism by which Liraglutide ameliorates hepatic steatosis in a high-fat diet (HFD)-induced rat model of NAFLD in vivo and in vitro.

Materials and methods: NALFD rat models and hepatocyte steatosis in HepG2 cells were induced by HFD and palmitate fatty acid treatment, respectively. AMPK inhibitor, Compound C was added in HepG2 cells. Autophagy-related proteins LC3, Beclin1 and Atg7, and AMPK pathway-associated proteins were evaluated by Western blot and RT-PCR.

Results: Liraglutide enhanced autophagy as showed by the increased expression of the autophagy markers LC3, Beclin1 and Atg7 in HFD rats and HepG2 cells treated with palmitate fatty acid. In vitro, The AMPK inhibitor exhibited an inhibitory effect on Liraglutide-induced autophagy enhancement with the deceased expression of LC3, Beclin1 and Atg7. Additionally, Liraglutide treatment elevated AMPK levels and TSC1, decreased p-mTOR expression.

Conclusions: Liraglutide could upregulate autophagy to decrease lipid over-accumulation via the AMPK/mTOR pathway.

1. Introduction

At present, non-alcoholic fatty liver disease (NAFLD) is prevalent and keeps rising. In developed countries, about 80% of adults with obesity or diabetes has been suffering from NAFLD [1,2], and the NAFLDs has relatively higher risk of liver fibrosis and liver cancer [3,4]. It is generally believed that NAFLD is closely related to insulin resistance, obesity, diabetes, dyslipidemia, and other metabolic syndromes that clinically manifest in the liver [5–7]. NAFLD includes simple fatty liver, fatty hepatitis, and fatty liver-related cirrhosis, which can develop into liver cancer [8]. NAFLD has also been considered as a high risk to induce cardiovascular and cerebrovascular diseases [9].

It is well understood that insulin resistance leads to excessive intake of free fatty acids (FFA) caused by steatosis. In addition, other factors, including oxidative stress injury, lipid peroxidation and abnormal cytokines, can cause local inflammatory changes that cause steatohepatitis in the hepatic lobules [10]. While this 'two-hit' hypothesis is currently widely accepted, NAFLD pathogenesis is not yet fully understood [11]. Excessive lipid deposition in the liver has been considered the primary factor that induces liver lipotoxicity [12–14]. Additionally, autophagy has been closely associated with intrahepatic lipid deposition. Previous studies have shown that autophagy degrades fat in hepatocytes as a relatively fixed process of auto-catabolism [15–17]. Autophagy can also promote "self-digestion" of accumulated, failing protein aggregates and defective organelles within the cell to maintain stability in the intracellular environment [18,19]. Lipid drops (LDs), which are biodegradation substrates, can be annexed and decomposed by the lysosomal pathway to maintain balanced intracellular lipid metabolism. Decreased intrahepatic autophagic activity, both *in vitro* and *in vivo*, can induce excessive lipid deposition [20].

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Evidence indicates that glucagon like peptide-1 (GLP-1) protects the liver from cell apoptosis induced by fatty acids though promoting autophagy and suppressing dysfunctional endoplasmic reticulum stress [21,22]. AMP-activated protein kinase (AMPK) is a cellular energy sensor that plays a key role in metabolic disorders and cancer [23]. The AMPK pathway is thought to critically regulate autophagy [24]. More specifically, AMPK activation can inhibit metabolic pathways and activate catabolic pathways to promote effective energy expenditure, which consequently improves how cells adapt to metabolic stress, resulting in increased cell survival [23,25].

Previous studies showed that GLP-1 improves hepatocyte steatosis by inducing autophagy through activating AMPK [26] in mice, suggesting that AMPK protects pancreatic β-cells from high glucose [27]. This evidence provides a new direction for targeting GLP-1 to prevent further deterioration of hepatic steatosis in NAFLD patients. However, the effect of GLP-1 analogs on high-fat diet (HFD)-induced NAFLD in rats has not been investigated. Further, the mechanism underlying liraglutide-induced autophagy is currently unknown. In the current study, we confirmed that liraglutide enhances autophagy in liver tissue and improves hepatic steatosis in a HFD-induced rat model of NAFLD. In addition, we provide evidence supporting the potential mechanism involving activation of the AMPK-mTOR pathway in liver homeostasis.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats ($n = 50$, 5 weeks of age, approximately 100 g) obtained from the Laboratory Animal Center of China Medical University were used to establish the NAFLD model. All rats were acclimated for one week prior to experimentation according to a previous protocol published [28]. Briefly, two initial groups of rats were randomly separated. The normal control (NC) group ($n = 13$) was fed chow containing 62% carbohydrates, 10% fat, and 28% protein; the HFD group ($n = 37$) was fed chow containing 34% carbohydrates, 52% fat, and 14% protein. Five rats from each group were randomly selected to confirm successful establishment of NAFLD after 12 weeks of feeding. (All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.)

Rats in the HFD group were randomly subdivided into four groups: HFD, 50 L, 100 L, and 200 L ($n = 8$ in each group). Rats in the HFD group were continually fed a HFD and were given 0.5 ml/kg of normal saline as needed. Rats in the 50 L, 100 L, and 200 L groups continued to receive HFD with different doses of liraglutide (L) (50, 100, or 200 μg/kg) (Victoza, Novo-Nordisk A/S, Denmark), respectively. The rats in the NC group ($n = 8$) continued to receive normal chow and were injected with 0.5 ml/kg of normal saline. Saline and liraglutide were injected subcutaneously at 8:00 am and 8:00 pm every day for 4 weeks.

2.2. Cell culture and treatment

HepG2 cells were cultured at 37 °C in a humidified chamber with 5% CO_2 and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco). There were a total of six cell culture groups: Control group treated with serum-free bovine serum albumin (BSA; Sigma–Aldrich, USA) medium; PA group treated with or without 400 M palmitate fatty acid (PA) for 24 h; and experimental groups treated with 400 M PA and either 10, 50, l00, or 500 nmol/L liraglutide for 24 h, respectively. These cells were divided into four groups: normal HepG2 cells (BSA), treated with PA (PA), treated with PA and liraglutide (100G), and Compound C for 30 min and then treated with PA and liraglutide (100G + C). Command C is an AMPK pathway inhibitor.

2.3. Western blot analysis

Liver specimens and whole-cell extracts were homogenized and centrifuged in RIPA buffer (Beyotime Institute of Biotechnology, China) containing a protease inhibitor cocktail with 1 mmol/L phenylmethanesulfonyl fluoride (Beyotime) on ice. Protein lysates were quantified using the BCA assay as previous described [28] (Pierce, USA). 50 μg of protein for each sample were separated by 8–10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% BSA and then incubated with primary antibodies against microtubule-associated protein LC3, Beclin1, AMPK, phosphorylated (p)-AMPK, TSC1, mTOR, and p-mTOR (all of which were used at 1:1000 and purchased from Cell Signaling Technology, USA) and GAPDH (used at 1:1000; Santa Cruz, CA, USA) at 4 °C overnight. The horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse secondary antibodies (1:5000, all from Santa Cruz Biotechnology, Inc.) were added at room temperature for 2 h after 3 washes (10 min each). The immunological complexes were visualized with Micro Chemi 4.2 (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel). Quantity One Software (Bio-Rad, USA) was used to quantify the protein band intensity.

2.4. Real-time RT-PCR

Total RNA from the frozen liver specimens (100 mg) and cell cultures were isolated using Trizol reagent (Takara Biotechnology Co., Ltd.). The PrimeScriptTM RT reagent kit was used to synthesize cDNA (Takara Biotechnology Co., Ltd., Dalian, China).

Real-time RT-PCR analysis was performed with a Thermal Cycler Dice Real Time detection System (Takara Bio Inc., Japan) using the SYBR Premix Ex Taq II kit (Tli RNaseH Plus) (Takara Biotechnology Co., Ltd.). Gene-specific primers for GFAP and GAPDH were purchased from Takara Biotechnology Co., Ltd. The following PCR protocol was used for all genes: reverse transcription step for 15 min at 37 °C, then denaturation at 85 °C for 5 s, 4 °C for 7 min, followed by an additional 40 cycles of amplification and quantification (5 s at 95 °C; 30 s at 60 °C; 30 s at 60 °C). mRNA expression was normalized to GAPDH as a housekeeping gene. The primers were designed (Primer Premier 5.0) and synthesized (Takara Biotechnology Co., Ltd. Dalian, China). For each gene, the relative change of mRNA in the samples was calculated by subtracting GAPDH Ct values from Ct values for the gene of interest using the $2-\Delta\Delta Ct$ method.

2.5. Electron microscopy

For transmission electron microscopy (TEM), liver specimens were fixed with 2.5% glutaraldehyde in a 0.1 M sodium cacodylate, pH 7.4, buffer at 4 °C and then minced into small (1 mm^3) fragments. After washing with 0.1 M phosphate buffered saline (PBS) three times for 15 min each, the liver tissue samples were fixed in 1% osmium tetroxide (OSO_4) for 1 h, followed by washes in 0.1 M PBS. Liver tissues were gradually dehydrated in ethanol solutions of 20%, 50%, 70% and 90% and embedded in Epon 812 epoxy resin before ultrathin sectioning. The ultrastructure of the sections was visualized using a JEM-1200EX electron microscope (JEOL Co., Japan) in the TEM laboratory of China Medical University.

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2.6. Hematoxylin and eosin (HE) Staining

The liver tissues were fixed at 5 mm from the edge of the right lobe of the liver and immersed in 4% paraformaldehyde. Then the tissues were dehydrated, waxed, embedded, and sliced for HE staining. Samples were dewaxed, benzenes were removed, hydrated, hematoxylin stained, washed, eosin stained, dehydrated, and imaged. According to the 2010 guidelines for the diagnosis and treatment of NLFD liver histopathological sections were scored based on the NAS system (0 to 8 points): (1) hepatocellular steatosis: 0 points (<5%); 1 point (5–33%); 2 points (34–66%); 3 points (>66%); (2) Inflammation within the lobules (inflammatory necrosis at 20-fold microscopy): 0 points (none), 1 point (<2), 2 points (2–4), 3 points (>4); (3) liver cell ballooning: 0 points, no; 1 point, rare; 2 points, more common.

2.7. Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and calculated with one-way analysis of variance (ANOVA) followed by a Newman–Keuls post-hoc test (SPSS 17.0). Results of comparisons were considered significantly different if the p value was < 0.05.

3. Results

3.1. Liraglutide improves hepatic histology during NAFLD development

After 16 weeks of HFD or normal chow diet we examined the histological features of the livers from NAFLD and NC mice. The liver surfaces were greyish yellow and the edges were blunt and thick in the HFD group compared to the NC group, in which the livers were bright red with sharp edges. Liraglutide dose-dependently ameliorated the pathological hepatic histology in the HFD group (Fig. 1A–E).

As shown in Fig. 2A–E, liraglutide improved the hepatic histology by significantly reducing both fatty droplets and inflammatory foci number. NAS quantification of liver sections further confirmed the beneficial effects of liraglutide treatment on hepatic histology (Table 1). Compared to the NC rats, the HFD rats had more

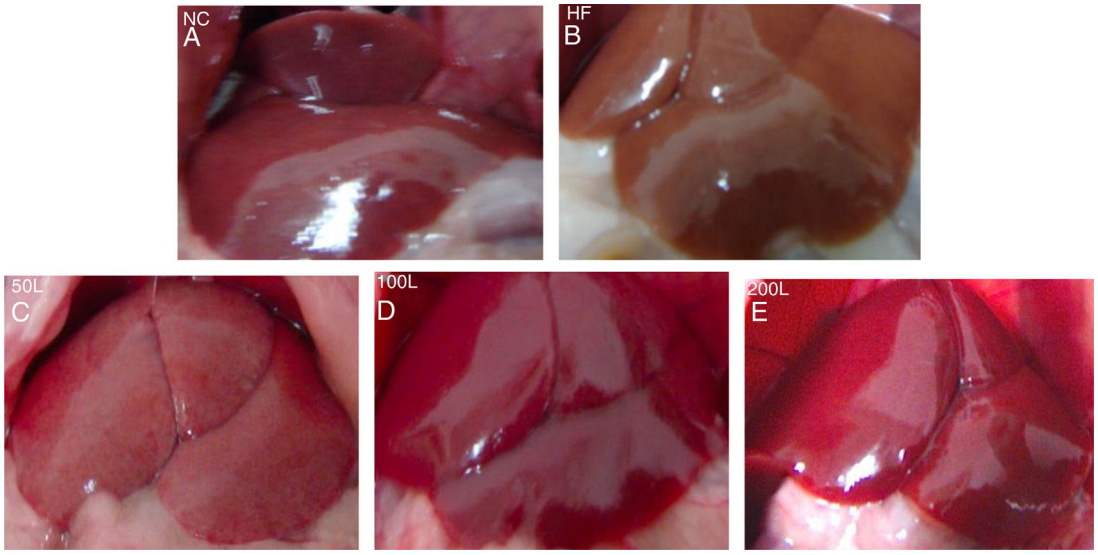

Fig. 1. The general changes in liver tissue after 16 weeks of HFD. A. NC group, B. HFD control group, C. 50 L group: low dose liraglutide intervention group (50 μg/kg), D. 100 L group: middle dose liraglutide intervention group (100 μg/kg), E. 200 L group: high-dose liraglutide intervention group (200 μg/kg).

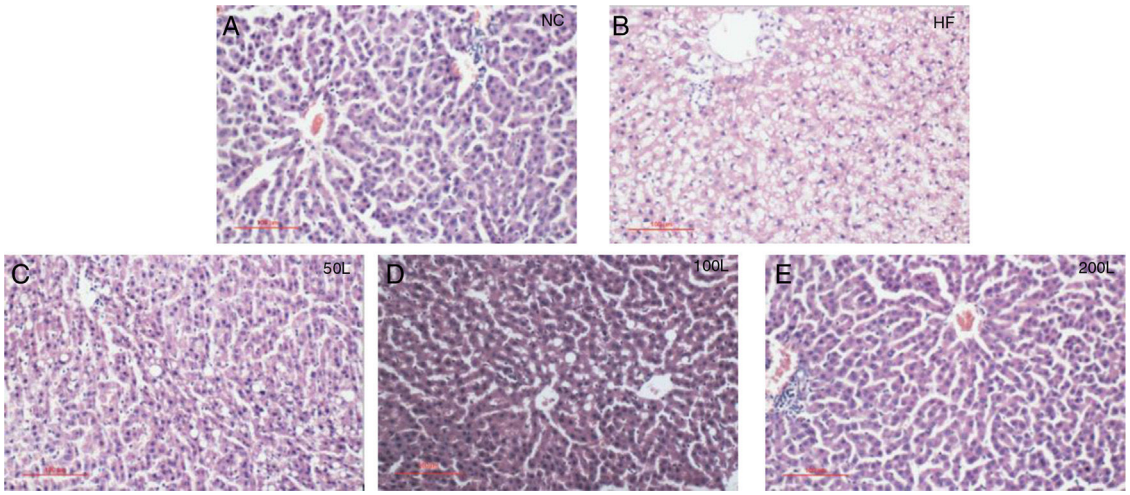

Fig. 2. Histopathological changes in rat livers 16 weeks after HFD. A. Histopathological changes (× 200); B. Rat liver histopathological NAS scores; C. 50 L group: low dose liraglutide intervention group (50 μg/kg), D. 100 L group: middle dose liraglutide intervention group (100 μg/kg), E. 200 L group: high-dose liraglutide intervention group (200 μg/kg).

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Table 1
The NAS scores of hepatic pathological sections after 16 weeks of HFD

Group	Steatosis	Inflammatory necrosis	Balloon-like changes
NC	0.28 ±0.48	0.29 ±0.48	0
HF	2.80±0.38*	1.85±0.38*	1.57±0.53*
50L	1.29±0	1.00±0.00#	0.85±0.38#
100L	1.13±0.53#	0.71±0.56#	0.42±0.54#
200L	0.37±0	0.25±0.52#	0.13±0.35#

* Compared to NC group, $P < 0.05$
# compared to HF group, $P < 0.05$

hepatocellular steatosis ($P < 0.05$), but liraglutide dose-dependently and significantly decreased steatosis, inflammation, and ballooning in the HFD group compared to the NC group ($P < 0.05$).

3.2. Liraglutide improves autophagy in the NAFLD rat model

We used electron microscopy to measure autophagosomes in each group. We found that the number of autophagosomes in the HFD group was significantly lower compared to the NC group. Liraglutide treatment dose-dependently increased the number of autophagocytic bodies in the HFD group compared to the NC group (Fig. 3A and B).

To further determine whether liraglutide improves autophagy in HFD-induced NAFLD rats, we measured the expression of autophagy-related proteins, LC3, Beclin1 and Atg7, in liver tissues. We found that LC3, Beclin1 and Atg7 mRNA levels and protein expression were significantly reduced in the HFD group and that liraglutide treatment dramatically increased expression of these autophagy markers (Fig. 4A–F).

3.3. Liraglutide improves autophagy in HepG2 cell in vitro

We measured mRNA and protein expression of the autophagy-related proteins, LC3, Beclin1 and Atg7, in HepG2 cells following PA treatment. In the PA group, mRNA and protein expression of LC3, Beclin1 and Atg7 were significantly lower compared to the BSA group ($P < 0.01$). However, liraglutide dose-dependently increased the mRNA and protein levels of LC3, Beclin1 and Atg7 relative to the PA group ($P < 0.01$) (Fig. 5A–F).

3.4. The effect of liraglutide on autophagy in HepG2 cells following AMPK pathway inhibition

To investigate whether the AMPK pathway was involved in liraglutide-mediated improvement of autophagy after PA treatment, we measured protein levels of LC3, Beclin1 and Atg7 in

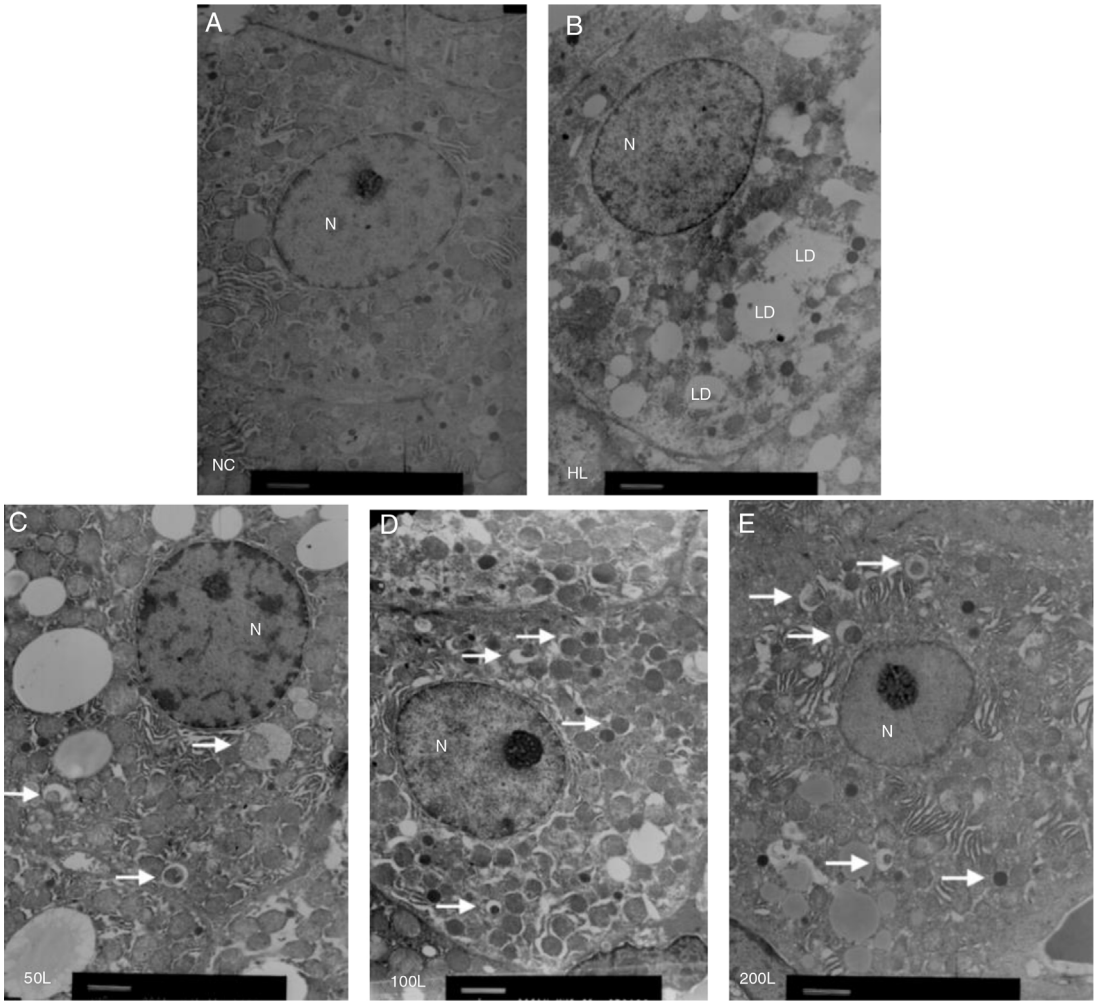

Fig. 3. Ultrastructure of hepatocytes observed by electron microscopy. A. NC group, B. HF group, C. 50 L group: low dose liraglutide intervention group (50 μg/kg), D. 100 L group: middle dose liraglutide intervention group (100 μg/kg), E. 200 L group: high-dose liraglutide intervention group (200 μg/kg). N, nucleus; LD, lipid droplet; →, autophagy body.

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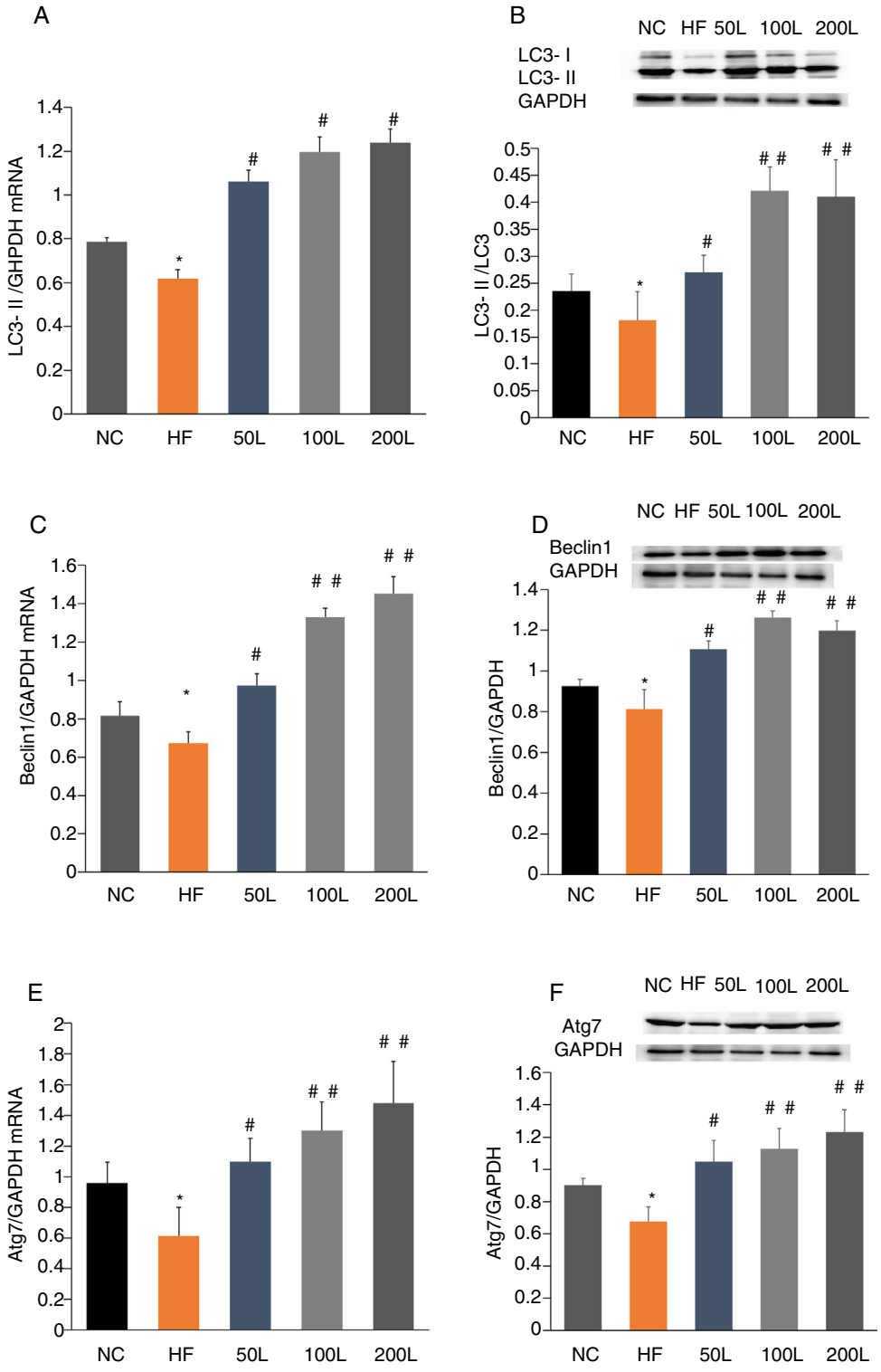

Fig. 4. Liraglutide reversed the decrease in autophagy related proteins in HFD-induce NAFLD rats. A and B. LC3II mRNA expression and LC3II/LC3I protein were assessed by real-time RT-PCR and Western blot, respectively. C and D. Beclin1 mRNA and protein expression were detected by real-time RT-PCR and Western blot, respectively. E and F. Atg7 mRNA and protein expression were measured by real-time RT-PCR and Western blot, respectively.

HepG2 cells. The results showed that the levels of LC3, Beclin1 and Atg7 were significantly higher in the liraglutide intervention group (100G group) compared to the PA group ($P < 0.01$). However, LC3, Beclin1 and Atg7 levels were significantly lower in the inhibition group (100G+C) containing Compound C, PA (400 mmol/L) and liraglutide (100 nmol/L), compared to the 100G group ($P < 0.01$) (Fig. 6A–C).

3.5. *Effects of liraglutide on AMPK pathway-associated proteins following AMPK pathway inhibition*

We measured expression of AMPK pathway-associated proteins to confirm that liraglutide-mediated autophagy induced by PA could be reversed by inhibiting the AMPK pathway. As shown in Fig. 6, the levels of p-AMPK/AMPK, TSC1, p-mTOR/mTOR were

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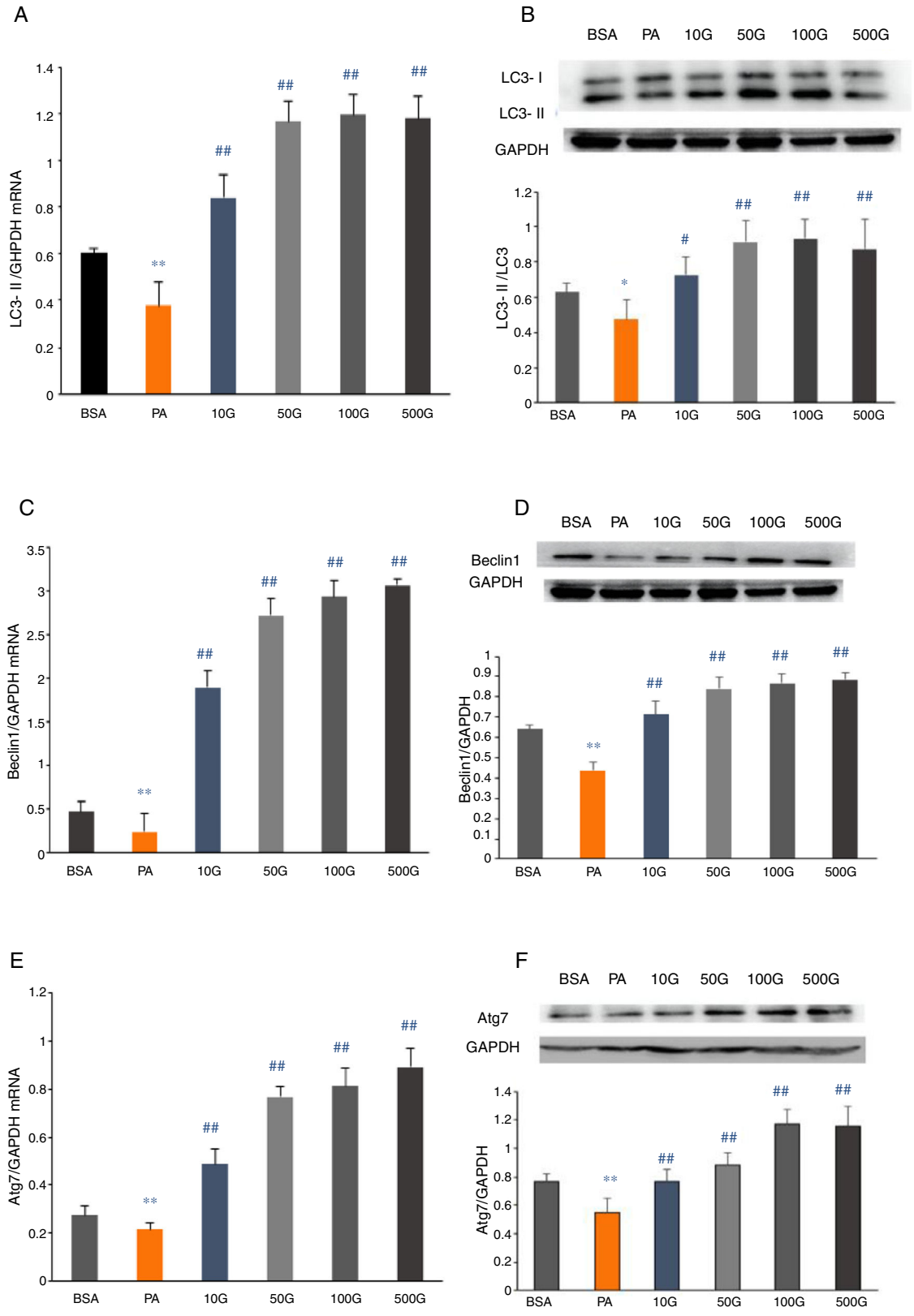

Fig. 5. Liraglutide reversed the decrease in autophagy related proteins in HepG2 cells. A and B. LC3II mRNA and protein expression were assessed by real-time RT-PCR and Western blot, respectively. C and D. Beclin1 mRNA and protein expression were detected by real-time RT-PCR and Western blot, respectively. E and F. Atg7 mRNA and protein expression were measured by real-time RT-PCR and Western blot, respectively.

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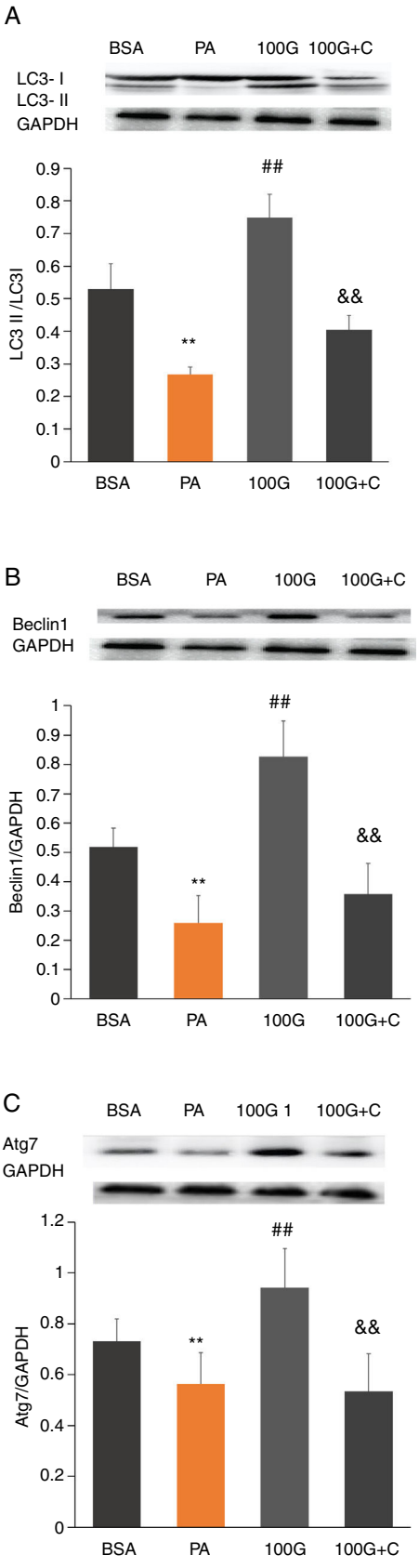

Fig. 6. The effect of liraglutide on autophagy in HepG2 cells in the presence of the AMPK pathway inhibitor. A. Expression of LC3II/LC3I detected by Western blot with or without the AMPK pathway inhibitor. B. Expression of Beclin1 detected by Western blot with or without the AMPK pathway inhibitor. C. Expression of Atg7 detected by Western blot with or without the AMPK pathway inhibitor.

decreased ($P < 0.01$) in the PA group (400 μmol/L PA) compared to the control group (BSA group). Furthermore, the levels of p-AMPK/AMPK, TSC1, and p-mTOR/mTOR were increased in the liraglutide intervention group (100G group) compared to the PA group ($P < 0.05$). The levels of p-AMPK/AMPK, TSC1, p-mTOR/mTOR were significantly lower than those in the 100G group compared to the inhibition group (100 G+C) containing Compound C, PA (400 μmol/L), and liraglutide (100 nmol/L) ($P < 0.01$) (Fig. 7A–C).

4. Discussion

The prevalence of NAFLD has increased worldwide, affecting both adults and children. Liraglutide, a GLP-1 analog, has been reported to decrease lipid deposition and inflammation in hepatocytes [29,30]. However, few studies have investigated whether liraglutide can improve hepatic lipid accumulation in NAFLD. In the current study, we used an established rat model of HFD-induced NAFLD and demonstrated that liraglutide significantly decreased the number of autophagosomes. Liraglutide treatment dose-dependently improved autophagy, which was confirmed by measuring expression of autophagy-related proteins *in vivo* and *in vitro*. More importantly, the AMPK pathway inhibitor, Compound C, reversed liraglutide's effects on autophagy following PA treatment *in vitro*, which suggests that liraglutide-induced autophagy is mediated by the AMPK signaling pathway.

Autophagy is an intracellular pathway that maintains normal cellular function by promoting turnover of long-lived proteins [31]. Studies have demonstrated that enhanced autophagy can rescue pancreatic β-cells from glucotoxicity, and inhibition of autophagy augments caspase-3 activation [32], suggesting that autophagy might protect against Type 2 Diabetes. Previous evidence indicated that autophagy is suppressed in the presence of hyper-insulinemia induced by HFD in mice [33], which was consistent with our results; however, we note that we used a different animal model in our study. Our results indicate that autophagy was significantly decreased in the NAFLD rat model, since the autophagy-related proteins, LC3, Beclin1 and Atg7, were significantly reduced at both the mRNA and protein levels. We also noticed that autophagy-related protein levels were decreased in palmitate-induced lipotoxicity in HepG2 cells *in vitro*, which mimics the pathogenic features of the NAFLD model *in vivo*.

Liraglutide, a GLP-1 analog, regulates β-cell mass via multiple pathways [34–36]. Liraglutide was proven to decrease lipid accumulation in the steatotic LO2 cell model [37] and protect pancreatic β-cells from high glucose by enhancing autophagy via AMPK [26]. It is also known that GLP-1 exerts protective effects on hepatic steatosis [38]. In the present study, we found that the general appearance, histopathological changes, and the number of autophagic bodies in the livers of NAFLD rats improved after liraglutide treatment. Further evidence showed that liraglutide does-dependently increased the expression of autophagy-related proteins in rats fed a HFD and in HepG2 cells treated with PA. These results suggest that GLP-1 ameliorates HFD induced NAFLD by activating autophagy.

Existing research shows that the AMPK/mTOR pathway regulates downstream signaling to trigger autophagy [39]. AMPK could negatively affect liraglutide-induced increases in cell viability and autophagy to protect insulin-1 pancreatic β-cells from glucotoxicity in rats [27]. In addition, AMPK/mTOR signaling was involved in hepatic lipid metabolism induced by GLP-1 [26].

Our data revealed that Command C, an AMPK pathway inhibitor, reversed the enhanced autophagy induced by liraglutide in HepG2 cells treated with PA, which suggests that liraglutide intervention activates AMPK and up-regulates autophagy. Thus, we conclude

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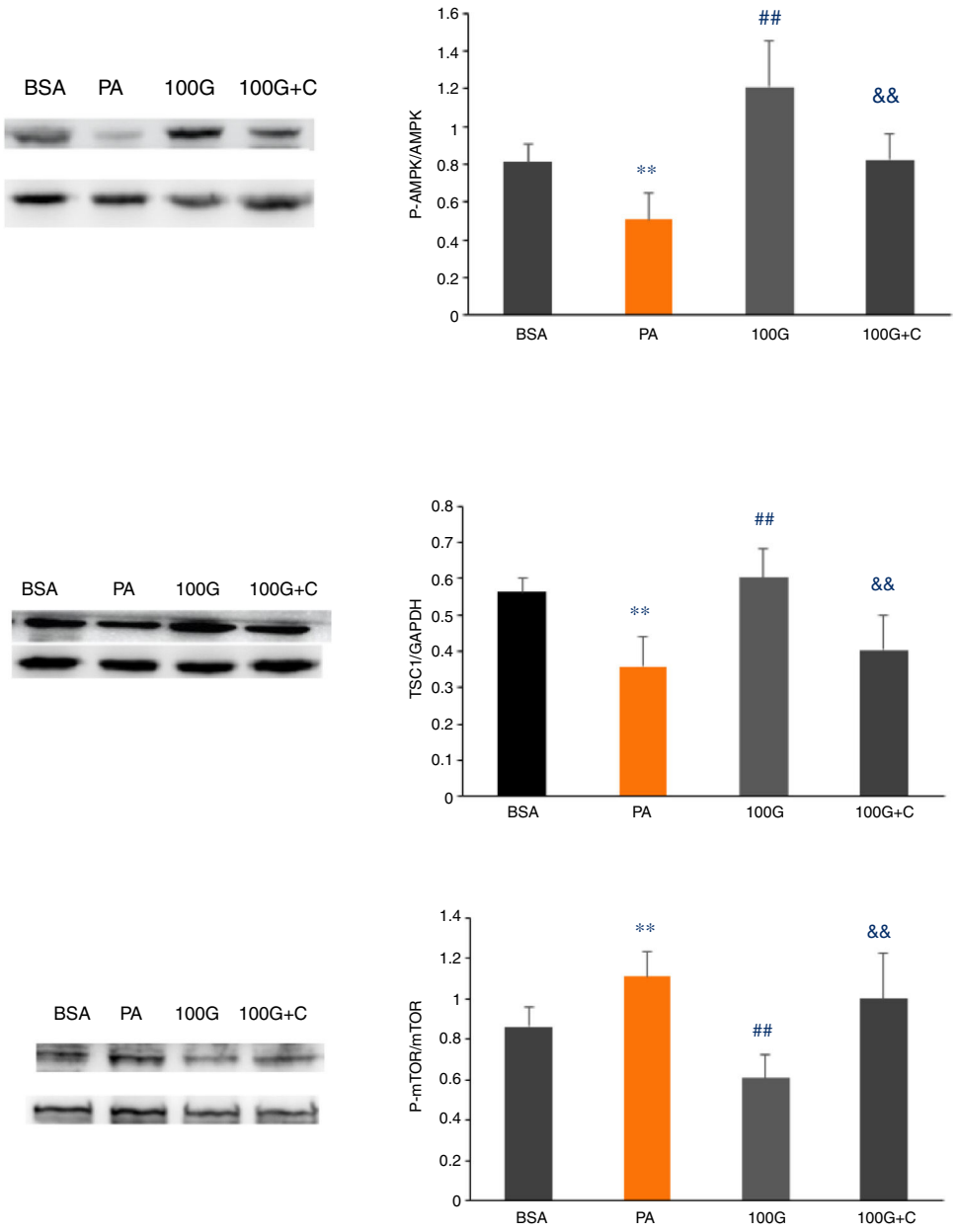

Fig. 7. Effects of liraglutide on AMPK pathway-associated proteins in the presence of the AMPK pathway inhibitor. A. Expression of p-AMPK/AMPK detected by Western blot. B. Expression of TSC1 detected by Western blot. C. Expression of p-mTOR/mTOR detected by Western blot.

that the AMPK pathway plays an important role in regulating autophagy induced by liraglutide.

In conclusion, the current study demonstrates that liraglutide can improve hepatic steatosis via activating the AMPK pathway. These data suggest that GLP-1 may play a protective role in several models of NAFLD and that modulation of AMPK could be a potential target for lipid metabolic disorders.

Abbreviations

AMPK	adenosine 5′-monophosphate (AMP)-activated protein kinase
Atg7	autophagy related gene 7
Beclin1	heterozygous disruption of beclin1
cAMP	cyclic adenosine monophosphate
GLP-1	glucagon-like peptide-1
LC3	micro-tubule-associated protein1 light chain 3
mTOR	the mammalian target of rapamycin
NAFLD	nonalcoholic fatty liver disease
PA	palmitate
T2DM	type 2 diabetes mellitus
TSC1	tuberous sclerosis-1

Author's contribution

Description of author roles in manuscript creation: Jian Du designed the experiment. Yini He, Na Ao and Jing Yang performed the performed experiments, Xiaochen Wang and Shi Jin processed the data, Yini He wrote the paper, and Jian Du modified the paper.

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Conflict of interest

All authors declare no conflicts of interest.

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