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Original Article

Palmitic acid induces autophagy in hepatocytes via JNK2 activation

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Aim: Free fatty acid-induced lipotoxicity plays a crucial role in the progression of nonalcoholic fatty liver disease (NAFLD). In the present study we investigated the effects of a high-fat diet and free fatty acids on the autophagic process in hepatocytes *in vivo* and *in vitro* and the underlying mechanisms.

Methods: LC3-II expression, a hallmark of autophagic flux, was detected in liver specimens from patients with non-alcoholic steatohepatitis (NASH) as well as in the livers of C57BL/6 mice fed a high-fat diet (HFD) up to 16 weeks. LC3-II expression was also analyzed in human SMMC-7721 and HepG2 hepatoma cells exposed to palmitic acid (PA), a saturated fatty acid. PA-induced apoptosis was detected by Annexin V staining and specific cleavage of PARP in the presence and absence of different agents.

Results: LC3-II expression was markedly increased in human NASH and in liver tissues of HFD-fed mice. Treatment of SMMC-7721 cells with PA increased LC3-II expression in time- and dose-dependent manners, whereas the unsaturated fatty acid oleic acid had no effect. Inhibition of autophagy with 3MA sensitized SMMC-7721 cells to PA-induced apoptosis, whereas activation of autophagy by rapamycin attenuated PA-induced PARP cleavage. The autophagy-associated proteins Beclin1 and Atg5 were essential for PA-induced autophagy in SMMC-7721 cells. Moreover, pretreatment with SP600125, an inhibitor of JNK, effectively abrogated PA-mediated autophagy and apoptosis. Specific knockdown of JNK2, but not JNK1, in SMMC-7721 cells significantly suppressed PA-induced autophagy and enhanced its pro-apoptotic activity; whereas specific knockdown of JNK1 had the converse effect. Similar results were obtained when HepG2 cells were tested.

Conclusion: JNK1 promotes PA-induced lipoapoptosis, whereas JNK2 activates pro-survival autophagy and inhibits PA lipotoxicity. Our results suggest that modulation of autophagy may have therapeutic benefits in the treatment of lipid-related metabolic diseases.

Keywords: hepatocytes; nonalcoholic fatty liver disease; free fatty acid; autophagy; apoptosis; palmitic acid; 3MA; rapamycin; SP600125; Beclin1; Atg5; JNK2

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in the majority of the developed world, and the incidence of NAFLD in many Asian countries, including China, Korea, Japan, and India, has risen rapidly in recent years[1]. NAFLD refers to a wide spectrum of liver disease from steatosis (fatty liver) to non-alcoholic steatohepatitis (NASH) and cirrhosis[2]. Therefore, NAFLD has been attracting more attention in the past few years. Free fatty acid (FFA)-induced lipotoxicity has been documented to play an essential role in the pathogenesis of diseases like obesity, diabetes, and NAFLD[3]. It is well established that FFAs can be classified chemically into two groups: saturated and unsaturated FFAs. Saturated FFAs, such as palmitic acid (PA) have greater lipotoxicity than unsaturated FFAs, such as oleic acid (OA)[4]. Nevertheless, the detailed mechanisms by which FFAs contribute to hepatic lipotoxicity remain unclear.

Autophagy has emerged as a homeostatic mechanism that regulates the turnover of long-lived or damaged proteins and organelles and helps to buffer a variety of cellular stresses

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by recycling intracellular constituents[5]. Recent studies have implicated the dysregulation of autophagy in the pathogenesis of a number of human diseases including cancer, infection, and degenerative and metabolic disorders[6]. It is well established that autophagy is strictly controlled by a group of autophagy-related genes (ATG genes), among which Atg8/LC3, Atg6/Beclin1, and Atg5 are the best characterized in mammalian cells[7].

Increasing evidence has shown that autophagy contributes to the physiological and pathological responses of cells to lipid stimulation. Using gene knockout mice, Singh *et al* verified the essential role of autophagy in the regulation of hepatic lipid stores[8]. Ding *et al* found that inhibition of autophagy exacerbates steatosis in mouse models of acute ethanol-induced hepatotoxicity[9], suggesting that autophagy plays a central role in regulating lipid metabolism and differentiation. Observations in pancreatic β-cells also indicate the protective effects of autophagy in maintaining normal cellular function[10]. However, the reported effects of FFAs on autophagy are still controversial[11–14]. The potential role of autophagy in FFA-induced NAFLD and its underlying molecular mechanisms remain to be elucidated.

Here, we demonstrate that PA, but not OA, triggers autophagy responses in hepatic cells via a mechanism involving the activation of JNK2, which antagonizes PA-JNK1 induced cytotoxic effects. These data suggest that the distinct activities of JNK1 and JNK2 are critical to liver homeostasis. A specific inhibitor of JNK1 may have more therapeutic benefits for PA-induced lipotoxicity, which might provide a potential new approach for the prevention and treatment of NAFLD.

Materials and methods

Patients samples

Liver specimens were obtained from hepatic hemangioma patients with or without NAFLD who received surgical resection in the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) from 2009 to 2010 with the approval of the Human Research Committee of Second Military Medical University and with the patients' consents.

Animals experiments

C57BL/6 mice were purchased from the Centre of Animal Model in Jiangsu Province, and housed in individual microisolator cages with free access to sterile water and irradiated HFD (2% cholesterol, 7% lard, 8.3% yolk, 16.7% sucrose, and 66% base forage) in a specific pathogen-free facility. Male mice (6–8 weeks) weighing 23–25 g were used in all experiments. All animals received human care according to the National Research Council's Guidelines. The grade of the animals is SPF. The given certificate number of the animal breeder is SCXK-2005-002.

Antibodies and reagents

The primary antibodies against LC3 (#2775), Beclin-1 (#3738), Atg5 (#2630), PARP (#9542), T-JNK (#9252), phospho-JNK (#9251), and GAPDH (#2118) were purchased from Cell Signaling Technology (CST, USA). Endotoxin-free bovine serum albumin (BSA), palmitic acid (PA), oleic acid (OA), 3-methyl adenine (3-MA), and rapamycin were from Sigma-Aldrich (USA). The pan-caspase inhibitor, ZVAD, the caspase 3 and 8 inhibitors (DEVD-fmk and IETD-fmk), and the JNK inhibitor (SP600125) were obtained from Calibiochem (USA). Hoechst 33342 was from the Beyotime Institute of Biotechnology (China).

Cell lines and cell culture

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SMMC-7721 cells were from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and were passed every 2–3 d to maintain logarithmic growth.

Fatty acid treatment

PA and OA were dissolved as described previously. Briefly, PA and OA were freshly diluted in 10% BSA from 100 mmol/L and 200 mmol/L stock solutions, respectively. Thereafter, 5 mmol/L PA or 2 mmol/L OA solutions were diluted to 500 μmol/L in pre-warmed DMEM (37 °C). The media in the culture was immediately replaced with media containing 500 μmol/L PA or 500 μmol/L OA and 1% BSA. Equal amounts of BSA were added to the control cells in each experiment, and lower concentrations of PA were diluted in 1% BSA to standardize the BSA concentration for all experiments.

Detection of green fluorescent protein-LC3 expression

A green fluorescent protein (GFP)-human LC3 fusion protein-expressing plasmid pEGFP-LC3 was kindly provided by Prof Mu-jun ZHAO (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). Transfection experiments were performed as described previously. Briefly, cells (5×10^4 per well) were seeded in six-well plates. After 24 h, cells were transfected with 2 μg of plasmid using polyethyleneimine (PEI) (Polyplus; AFAQ). GFP fusion proteins were observed under a laser scanning microscope system (Olympus). Cells with more than five GFP-LC3 punctate dots were considered positive and counted. The percentage of GFP-LC3-positive cells with GFP-LC3 punctae was determined from three independent experiments.

Western blotting analysis

Western blotting was performed as described previously. Briefly, whole-cell extracts or tumor specimens were prepared in lysis buffer [Tris-HCl (20 mmol/L), pH 7.4, NaCl (150 mmol/L), glycerol (10%), Nonidet P-40 (0.2%), EDTA (1 mmol/L), EGTA (1 mmol/L), PMSF (1 mmol/L), NaF (10 mmol/L), aprotinin (5 mg/mL), leupeptin (20 mmol/L), and sodium orthovanadate (1 mmol/L)] and centrifuged at 12 000×*g* for 15 min. Protein concentrations were measured using the BCA assay (Pierce). Immunoblotting was performed

using specific primary antibodies, and immunocomplexes were incubated with the appropriate fluorescein-conjugated secondary antibody and then detected using an Odyssey fluorescence scanner (Li-Cor, Lincoln, NE, USA).

Cell death assay

Nuclear staining with Hoechst 33342 was applied to detect nuclear fragmentation characteristic of apoptosis as described previously. Treated cells were stained and examined under fluorescent microscopy. At least 500 cells were counted.

Transient small interfering RNA (siRNA) transfection

The nonspecific siRNA oligonucleotides and siRNA oligonucleotides targeting human Beclin1 and Atg5 were purchased from Invitrogen. SMMC-7721 and HepG2 cells were transfected using INTERFERin transfection reagent (Polyplus) according to the manufacturer's protocol as described previously.

Statistical analysis

Numeric results are expressed as mean±SD. Statistical evaluation was carried out by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A value of $P<0.05$ was considered to be statistically significant.

Results

Autophagy is enhanced in human non-alcoholic steatohepatitis and the livers of mice fed a high-fat diet

To determine the existence and levels of autophagy in patients with non-alcoholic steatohepatitis (NASH), LC3-II expression, which has been widely used as a hallmark of autophagic flux, was assessed in human liver specimens. As shown in Figure 1A, protein levels of LC3-II were significantly increased in NASH liver tissues in comparison with normal controls. These results verify the association of increased autophagy levels in livers from NASH patients.

To further determine the effects of HFD on steatosis and autophagy, C57BL/6 mice were fed a normal diet (ND) or a HFD over a period of up to 16 weeks. Steatosis was found in livers of mice fed with a HFD (Supplementary Figure S1A). Consistent with the clinical finding, LC3-II protein levels were much higher in liver tissues from HFD-fed mice than those from ND-fed mice at all time intervals (Figure 1B), suggesting an increased number of autophagosomes in HFD-fed mouse livers. To confirm these observations, TEM studies were performed. As shown in Supplementary Figure S1B, both the number and size of the hepatic lipid droplets (LD), as well as the formation of autophagosomes, were markedly increased in the livers of mice fed a HFD for over 8 weeks. In contrast, autophagosome-like vacuoles were hardly seen in ND-fed mice. Taken together, these results confirm that autophagy is activated both in livers of NASH patients and mice fed with a HFD.

PA, but not OA, induces autophagy *in vitro*

Elevated serum FFA levels have been shown to correlate with the severity of NASH[15]. Recent work has demonstrated that saturated fatty acids such as PA are more cytotoxic compared to unsaturated fatty acids, such as OA[16]. To further confirm the above *in vivo* data and to dissect the potential effect of PA and OA on autophagy induction, we tested autophagy levels in SMMC-7721 (a human hepatoma cell line) following exposure to OA or PA. As shown in Supplementary Figure S1C, the induction of PA, but not OA, resulted in a significant increase in the levels of LC3-II for up to 12 h. In addition, treatment of SMMC-7721 cells with PA caused an obvious increase of LC3-II conversion in both a time- and dose-dependent manner (Figure 1C). Moreover, the total cellular expression levels of p62 also showed gradient decrease in response to PA treatment. Similar results were obtained for HepG2 cells (Supplementary Figure S2A). Furthermore, transfected LC3-GFP protein displayed a diffuse pattern in BSA-treated SMMC-7721 and HepG2 cells, while exposure of cells to PA resulted in a green punctate staining, indicating the accumulation of LC3-II within the autophagosomal membranes. As expected, fewer GFP-LC3 punctae were observed in cells treated with OA (Figure 1D and Supplementary Figure S2B).

Electron microscopy analysis verified that exposure of SMMC-7721 cells to PA resulted in the formation of autophagosomes and autophagic vacuoles, whereas autophagosome-like vacuoles were hardly seen in BSA-treated cells (Figure 1E). Similar observations were observed in HepG2 cells (Supplementary Figure S2C). These results demonstrate that saturated fatty acid induces autophagy in two different hepatoma cell lines.

Autophagy attenuates PA-induced apoptosis

Recent studies have documented that autophagy may serve as a pro-survival mechanism to protect cells from various types of cellular stress[6, 9, 10, 12, 13]. To determine whether autophagy plays a functional role in FFA-induced lipotoxicity, we examined the effects of PA-induced autophagy on apoptotic signaling in hepatoma cells. Consistent with several earlier observations[16], treatment of SMMC-7721 cells with PA activated cell death in a dose- and time-dependent manner, as indicated by the Annexin V staining and specific cleavage of PARP (Figure 2A and 2B). PA-induced cell death in SMMC-7221 and HepG2 cells was accompanied by chromatin condensation, a well-known marker of apoptotic cell death signaling (Supplementary Figure S3A). To determine whether autophagy affects PA-induced apoptosis, we modulated the autophagic pathway using specific pharmacologic agents. 3-MA, a pharmacological suppressor of the upstream PI3K/Beclin 1-Vps34 complex, inhibited PA-induced autophagy in SMMC-7221 cells, as indicated by its ability to inhibit PA-induced LC3-II accumulation, and simultaneously led to an increase in apoptosis as indicated by enhanced PARP cleavage levels and Annexin V positive cell number (Figure 2C). A significant increase in PA-induced chromatin condensation was also observed upon treatment with 3-MA, confirming its ability to enhance PA-induced apoptotic cell death (Supplementary Figure S3B). Conversely, induction of autophagy by rapamycin, which sup-

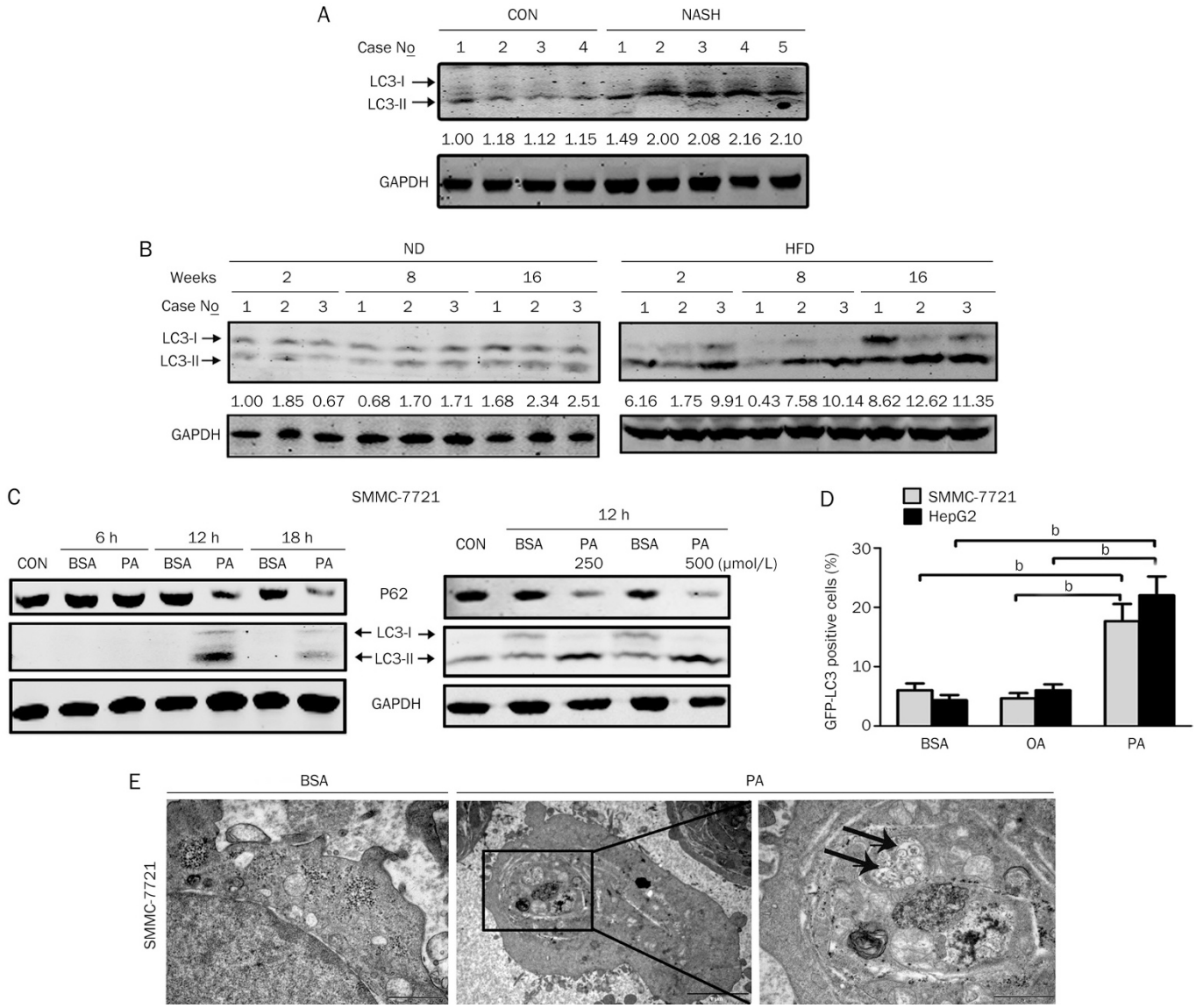

Figure 1. Autophagy is activated in human non-alcoholic steatohepatitis, in high-fat diet mouse liver and in PA-treated hepatic cells. (A) Liver specimens from control subjects (CON) or patients with non-alcoholic steatohepatitis (NASH) were homogenized, and the protein levels of LC3 were determined by Western blotting assay. GAPDH levels were also assessed as a loading control. The intensity of the protein bands was evaluated using Odyssey infrared imaging system application software, and the ratio of LC3-II intensity to GAPDH intensity was determined first, then the relative ratio of other samples to the far left sample was determined. (B) C57BL/6 mice were fed with a normal diet (ND) or a high fat diet (HFD) over a period of up to 16 weeks. Livers from ND or HFD-fed mice were homogenized, and the protein levels of LC3 and GAPDH were determined by Western blotting assay. (C) SMMC-7721 cells were left untreated (CON) or were treated with BSA or palmitic acid (PA), either at a fixed 500 μmol/L dose over a time-course (left panel) or at a fixed 12 h time point with varying doses (right panel). Protein levels of LC3, P62, and GAPDH in cell lysates were determined by Western blotting assay. (D) SMMC-7221 and HepG2 cells were transfected with GFP-LC3 plasmid for 24 h followed by exposure to BSA, oleic acid (OA, 500 μmol/L) or PA (500 μmol/L) for an additional 12 h. GFP-LC3 punctate formation was quantified by fluorescence microscopy of >500 cells. Results represent the mean±SD of data from three independent experiments. $^{b}P<0.05$. (E) SMMC-7721 cells were treated with BSA or PA (500 μmol/L) for 12 h followed by assessment by transmission electron microscopy. Representative results are shown. Magnification, ×5000 (left and middle panel) and ×20 000 (right panel).

presses mTOR, effectively prevented PA-induced cell apoptosis (Figure 2D) and chromatin condensation (Supplementary Figure S3C), further suggesting that PA-induced autophagy may have a pro-survival function during lipotoxic stress.

To determine whether the induction of autophagy by PA is dependent on apoptosis, we assessed the effects of a panel of apoptosis inhibitors on LC3 accumulation. Notably, pretreatment of SMMC-7721 cells with the pancaspase inhibitor ZVAD-fmk, the caspase 3 inhibitor DEVD-fmk or the caspase 8 inhibitor IETD-fmk attenuated PA-induced PARP cleavage, but had virtually no effect on the accumulation of LC3-II induced by PA (Figure 2E). This indicates that PA-induced

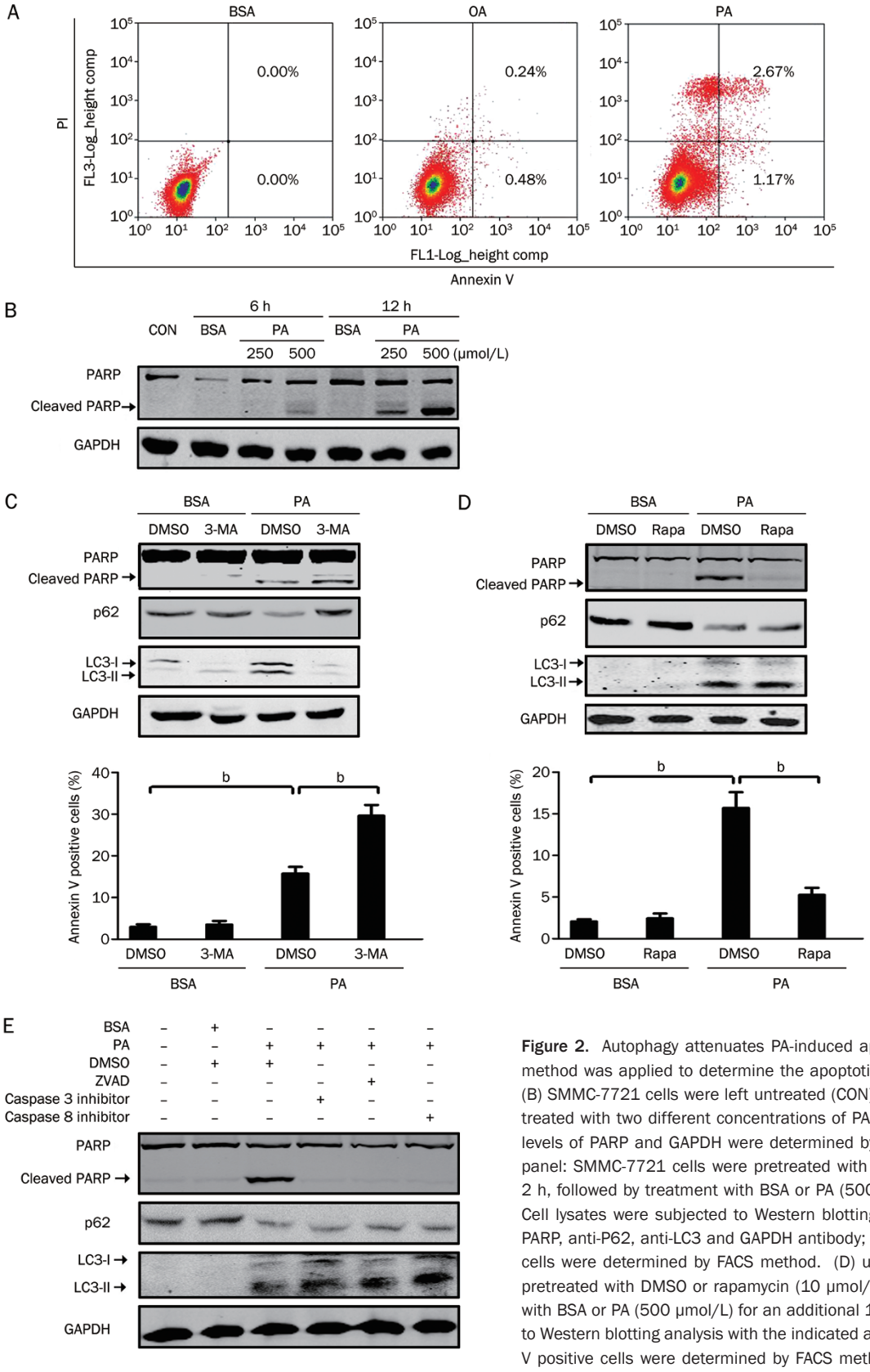

Figure 2. Autophagy attenuates PA-induced apoptosis. (A) Annexin V staining method was applied to determine the apoptotic cells after OA or PA treatment. (B) SMMC-7721 cells were left untreated (CON), were treated with BSA, or were treated with two different concentrations of PA for the indicated times. Protein levels of PARP and GAPDH were determined by Western blotting assay. (C) up panel: SMMC-7721 cells were pretreated with DMSO or 3-MA (10 mmol/L) for 2 h, followed by treatment with BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were subjected to Western blotting analysis and probed with anti-PARP, anti-P62, anti-LC3 and GAPDH antibody; bottom panel: Annexin V positive cells were determined by FACS method. (D) up panel: SMMC-7721 cells were pretreated with DMSO or rapamycin (10 μmol/L) for 2 h, followed by treatment with BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were subjected to Western blotting analysis with the indicated antibodies; bottom panel: Annexin V positive cells were determined by FACS method. (E) SMMC-7721 cells were pretreated with the pan-caspase inhibitor ZVAD, the caspase 3 inhibitor DEVD-fmk or the caspase 8 inhibitor IETD-fmk for 2 h, followed by exposure to BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were subjected to Western blotting with the indicated antibodies.

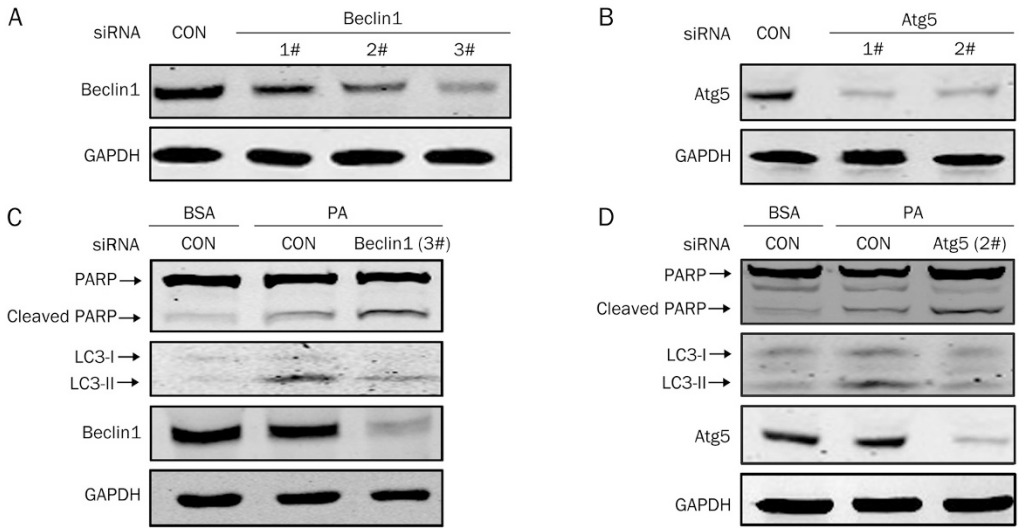

Figure 3. Beclin1 and Atg5 are required for PA-induced autophagy. (A and B) A control siRNA (CON) and three siRNAs targeting Beclin1 (A) or Atg5 (B) were transfected into SMMC-7721 cells for 36 h followed by exposure to PA (500 μmol/L) for an additional 12 h. Cell lysates were harvested, and expression of Beclin1 and Atg5 was analyzed by Western blotting assay to verify knockdown. GAPDH was also tested as a loading control. (C) SMMC-7721 cells were transfected with control siRNA (CON) or Beclin1 siRNA (3#) for 60 h, followed by exposure to BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were harvested and expression of PARP, Beclin1, LC3 and GAPDH was analyzed by Western blotting assay. (D) SMMC-7721 cells were transfected with control siRNA (CON) or Atg5 siRNA (2#) for 60 h, followed by exposure to BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were subjected to Western blotting with the indicated antibodies.

autophagy primarily functions upstream of apoptotic signaling to suppress apoptosis.

Beclin1 and Atg5 are required for PA-induced autophagy and inhibition of apoptosis

Because autophagy is tightly controlled by a group of autophagy-related genes (ATG genes)[17], we examined whether PA-induced autophagy is dependent on the essential Atg proteins, Beclin1 and Atg5. We used siRNA-mediated knockdown to specifically silence these genes. As shown in Figure 3A and 3B, 3# siRNA of Beclin1 and 2# siRNA of Atg5 showed the most significant gene silencing effects (more than 70% knock down efficiency), and, therefore, these siRNAs were employed in subsequent loss-of-function studies. PA-induced LC3-II accumulation was significantly prevented by Beclin1 knockdown, verifying the requirement of Beclin1 in PA-induced autophagy. Furthermore, higher levels of PARP cleavage were observed in Beclin1 siRNA-treated SMMC-7721 cells, confirming the essential role of Beclin1-mediated autophagy in cell survival in response to lipotoxic stress (Figure 3C). Similar results were observed for Atg5 siRNA-treated SMMC-7721 cells (Figure 3D). Taken together, these findings suggest that PA-induced autophagy suppresses apoptosis via a Beclin1 and Atg5-dependent pathway.

JNK activation mediates PA-induced autophagy

Autophagy is tightly controlled through diverse signaling pathways[7, 18]. To identify the signaling mechanisms by which PA induces autophagic responses, we examined the activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) by PA in SMMC-7221 cells. Exposure to PA led to enhanced phosphorylation of both of these signaling mediators. The ERK-specific inhibitor U0126 and the p38-specific inhibitor SB202190 blocked ERK and p38 phosphorylation, but did not prevent PA-induced LC3-II accumulation (Supplementary Figure S4A and S4B). These findings rule out the possibility that PA induces autophagy through an ERK- or p38-dependent mechanism. Two widely used antioxidants, NAC and GSH, also failed to block the LC3-II accumulation in PA-treated SMMC-7721 cells (Supplementary Figure S4C), indicating that PA-induced autophagy is not regulated through signaling by reactive oxygen species.

Activation of c-Jun N-terminal kinase (JNK) has also been reported to be associated with the development of NASH[19]. Consistent with previous reports, much higher levels of phospho-JNK were observed in liver tissues from HFD-fed mice than ND-fed mice (Figure 4A). As the JNK pathway has been documented to play an important role in the regulation of autophagy[20], we tested whether PA-induced autophagy requires JNK activation. PA treatment resulted in significant activation as compared to BSA-treated cells for both SMMC-7221 and HepG2 cells. Moreover, pretreatment with SP600125, a JNK-specific pharmacological inhibitor, blocked JNK activation and effectively abrogated LC3-II accumulation in a dose-dependent manner in both cell lines (Figure 4B). To verify these results, we examined the effects of SP600125 on the formation of punctate in SMMC-7721 and HepG2 cells transfected with LC3-GFP plasmid. SP600125 significantly reduced the visible green dots in the PA-treated cells and redistributed

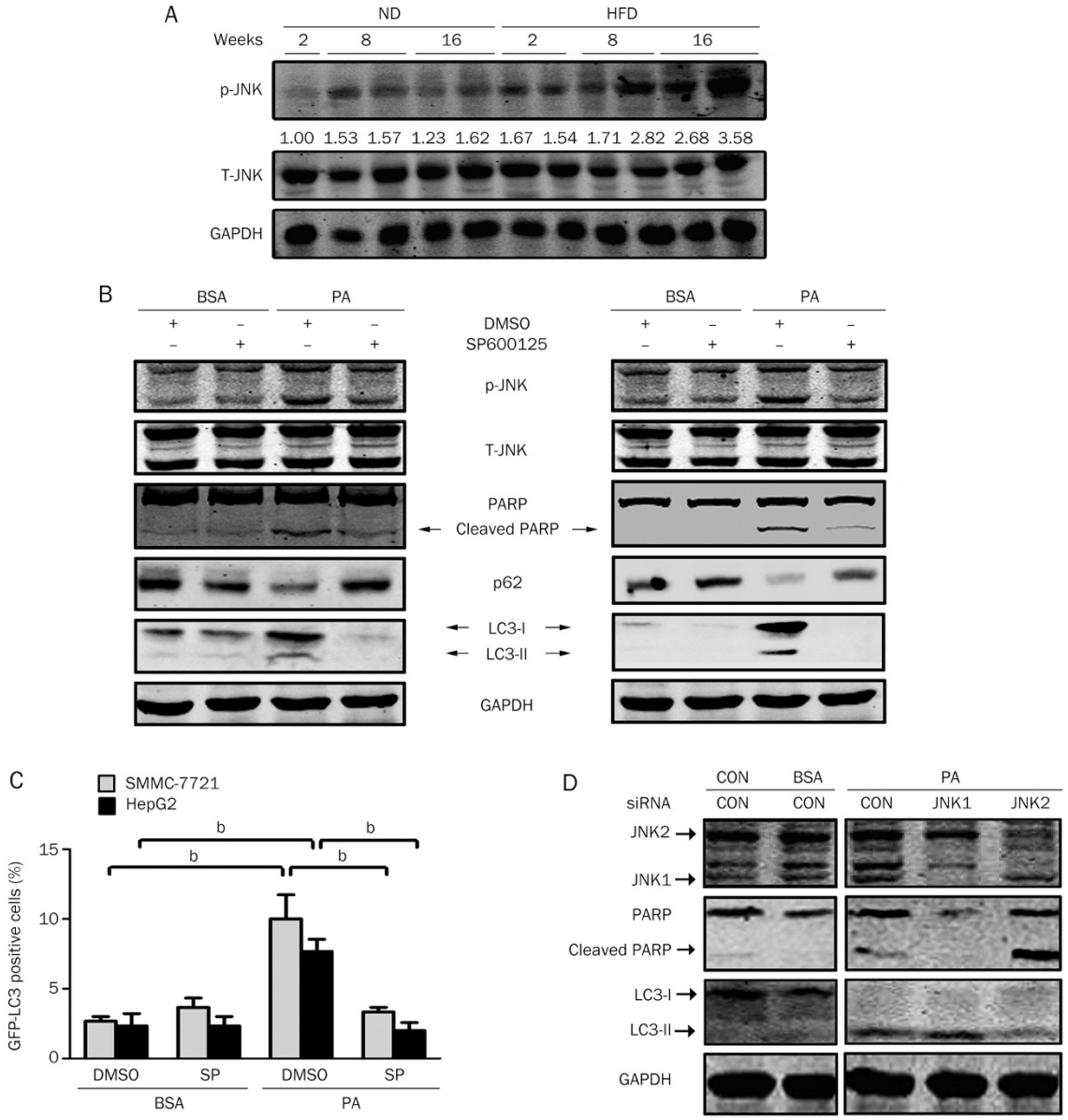

Figure 4. JNK1 and JNK2 activation differentially regulates PA-induced autophagy and apoptosis. (A) C57BL/6 mice were fed a ND or a HFD over a period of up to 16 weeks. Livers from ND or HFD-fed mice were homogenized, and the protein levels of phosphorylated JNK (p-JNK), total JNK (T-JNK) and GAPDH were determined by Western blotting assay. (B) SMMC-7221 cells (left panel) and HepG2 cells (right panel) were pretreated with DMSO or increasing concentrations of the JNK inhibitor SP600125 for 2 h, followed by treatment with BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were harvested, and the expression of p-JNK, T-JNK, PARP, p62, LC3, and GAPDH was determined by Western blotting assay. (C) Cells were pretreated with DMSO or SP600125 (10 μmol/L) for 2 h, followed by treatment with BSA or PA (500 μmol/L) for an additional 12 h. The percentage of cells positive for GFP-LC3 punctae formation was quantified by fluorescence microscopy. Results represent the mean±SD of data from three independent experiments. $^{b}P<0.05$. (D) SMMC-7721 cells were transfected with control siRNA (CON), JNK1 siRNA or JNK2 siRNA for 60 h, followed by treatment with BSA or PA (500 μmol/L) for an additional 12 h. Cell lysates were harvested, and the expression of JNK1, JNK2, PARP, LC3, and GAPDH was evaluated by Western blotting assay.

GFP-LC3 into the cytoplasm (Figure 4C and Supplementary Figure S4D). These results confirm that PA-induced autophagy is mediated by the JNK pathway, but is independent of ERK and p38.

JNK1 and JNK2 differentially regulate PA-induced autophagy and apoptosis

It has been well recognized that JNKs are implicated in apoptosis control, and we therefore sought to determine whether

JNK activation is responsible for the lipoapoptosis that occurs concurrently with PA treatment. Interestingly, SP600125 pretreatment substantially inhibited PA-dependent activation of PARP cleavage (Figure 4B). This suggests that although PA-induced autophagy functions to oppose the associated apoptosis induction (Figure 2), both pathways are positively regulated by JNK activation.

Because recent studies have demonstrated a complicated reciprocal regulation between JNK1 and JNK2, we wondered whether these two forms of JNK might have distinct effects on autophagy and apoptosis in response to PA treatment. Intriguingly, siRNA-mediated gene silencing of JNK1 almost completely blocked the PA-induced apoptosis, as evidenced by reduced levels of PARP cleavage, but had virtually no effect on LC3 protein levels. In contrast, specific JNK2 knockdown attenuated PA-induced autophagy and profoundly enhanced the pro-apoptotic effect of PA, as evidenced by the decreased LC3-II accumulation and increased PARP cleavage (Figure 4D). These data suggest that JNK1 promotes PA-induced lipoapoptosis, whereas JNK2 inhibits lipoapoptosis, at least partly by activating pro-survival autophagy signaling.

Discussion

FFA-induced hepatic lipotoxicity has been shown to be closely related to the progression of NAFLD[3]. However, the mechanisms by which hepatocytes tolerate lipotoxicity remain largely unknown. Recently, autophagy has been implicated in the regulation of lipid metabolism[8] and lipotoxicity[10–13]. Nevertheless, reports regarding the effects of different FFA profiles on autophagy remain controversial. In the present study, we provided evidence that autophagy is activated both in human NASH livers and in HFD-fed mice. This effect is shown to be mediated in hepatoma cells by saturated PA, but not unsaturated OA. The latter finding is generally consistent with observations in multiple cell types including hepatocytes, pancreatic β-cells, skeletal muscle cells and endothelial cells, where saturated FFAs are more toxic than unsaturated FFAs[16]. On the other hand, Mei *et al* reported that OA but not PA had the ability to induce autophagy in hepatocytes[11]. These conflicting results may stem from several factors, including but not limited to the cell types and the concentration and duration of FFA treatment. Nevertheless, our results indicate that different FFAs have distinct toxic potential and that the FFA profile in serum or liver tissue could potentially be used to evaluate liver injury clinically.

Although autophagy has been recognized as a key cytoprotective mechanism to counteract various stress conditions, such as nutrient or growth factor deprivation, hypoxia, reactive oxygen species, damaged protein or organelles, high levels of autophagic activity can also lead to cell death. Interestingly, autophagy was also observed in mice fed a normal diet for 8 weeks or 16 week (Figure 1B), which might result from the relative low FFA content in normal diet. Since we did not observe the elevated serum ALT or AST concentration in the ND group in our previous study, we postulated that such an autophagy process emerges as a physiologically homeostatic mechanism, regulating the turnover of long-lived or damaged proteins and organelles and helping to buffer a variety of cellular stresses by recycling intracellular constituents. Our results strongly support the pro-survival role of autophagy in response to PA stimulation, which is consistent with an earlier report that autophagy retains a protective function in MEF cells treated with PA[12]. It is proposed that autophagy can degrade and clear accumulated diacylglycerol from the cells via lipases[12], thereby promoting cell survival in response to FFA stress. However, the detailed mechanism by which autophagy protects against FFA-mediated cytotoxicity needs further investigation.

Signaling pathways related to autophagy may differ depending on cell state. Although we observed significant activation of MAPKs, including ERK, p38, and JNK in the presence of PA, inhibitors of ERK or p38 failed to block LC3-II accumulation and p62 degradation, which argues against the possibility of a primary role for these two pathways in PA-induced autophagy. In contrast, our experiments identified a relationship between LC3-II accumulation and JNK activation: JNK pharmacological inhibitor SP600125 significantly attenuated the increased LC3-II expression in PA-treated cells. SP600125 also caused a reduction in PARP cleavage, suggesting that JNK plays a central role in FFA-triggered cytotoxicity by modulating both the autophagic and apoptotic processes. The dual role of the JNK pathway is seemingly inconsistent with the pro-survival role of autophagy in our model. However, we provide evidence that the two JNK isoforms may have differential functions in FFA-mediated autophagy and lipotoxicity: JNK1 promotes PA-induced lipoapoptosis, whereas JNK2 activates pro-survival autophagy and inhibits PA lipotoxicity.

At present, the molecular mechanisms of PA in triggering the activation of JNK and JNK2-mediated autophagy are still largely unknown. It has been documented that Protein Kinase C (PKC) μ acts upstream of JNK. Additionally, Tan *et al* established the role of PKC-α in PA-induced autophagy in MEF cells[12]. These observations highlight the potential importance of the PKC family in modulating FFA-triggered cell responses. Therefore, it would be of interest to investigate whether specific PKCs may mediate JNK2 activation under situations of FFA stress. Our studies here suggested that these two JNK molecules may serve as the key pair of modulators involved in the maintenance of cell homeostasis upon FFA stimulation. Based on the differential roles of JNK1 and JNK2, specific interference with JNK1 expression might have more therapeutic benefits than non-specific anti-JNK therapy for FFA-induced lipotoxicity, providing a potential therapeutic strategy to mitigate lipoapoptosis to prevent the progression of NAFLD.

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Author contribution

Qian-qian TU, Lei CHEN, Juan LI, Liang HU, Liang LI, and Min-hong LI designed and performed some of the experiments; Ruo-yu WANG and Dan-dan HUANG collected the human samples and prepared cell lines; Lei CHEN, Juan LI and Liang HU performed the data analysis; Rui-ying ZHENG and Yan-xin CHANG performed the ELISA, Western blotting, and RT-PCR experiments; Qian-qian TU, Lei CHEN, Meng-chao WU and He-ping HU designed the experiments and wrote the paper; and Hong-yang WANG provided supervision and modified the paper.

Abbreviations

FFA, free fatty acid; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PA, palmitic acid; OA, oleic acid; ATG, autophagy-related genes; LC3, microtubule-associated protein light chain 3.

Supplementary information

Supplementary Figures are available in the website of Acta Pharmacologica Sinica.

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