Autophagy activation by Jiang Zhi Granule protects against metabolic stress-induced hepatocyte injury

Yi-Yuan Zheng, Miao Wang, Xiang-Bing Shu, Pei-Yong Zheng, Guang Ji

AIM
To elucidate the potential role of autophagy and the protective effects of Jiang Zhi Granule (JZG) in metabolic stress-induced hepatocyte injury.

METHODS
An in vitro and in vivo approach was used in this study. HepG2 cells were incubated in culture medium containing palmitate (PA; 0, 0.1, 0.2, 0.3, 0.4 or 0.5 mmol/L) and treated with or without JZG (100 μg/mL) for 24 h or 48 h, and the progression of autophagy was visualized by stable fluorescence-expressing cell lines LC3 and p62. Western blot analyses were performed to examine the expression of LC3-II/LC3-I, p62, mTOR and PI3K, while mitochondrial integrity and oxidative stress were observed by fluorescence staining of JC-1 and reactive oxygen species. C57BL/6 mice
were divided into three groups: control group (n = 10), high fat (HF) group (n = 13) and JZG group (n = 13); and, histological staining was carried out to detect inflammation and lipid content in the liver.

RESULTS
The cell trauma induced by PA was aggravated in a dose- and time-dependent manner, and hepatic function was improved by JZG. PA had dual effects on autophagy by activating autophagy induction and blocking autophagic flux. The PI3K-AKT-mTOR signaling pathway and the fusion of isolated hepatic autophagosomes and lysosomes were critically involved in this process. JZG activated autophagy progression by either induction of autophagosomes or co-localization of autophagosomes and lysosomes as well as degradation of autolysosomes to protect against PA-induced hepatocyte injury, and protected mitochondrial integrity against oxidative stress in PA-induced mitochondrial dysfunction. In addition, JZG ameliorated lipid droplets and inflammation induced by HF diet in vivo, leading to improved metabolic disorder and associated liver injury in a mouse model of non-alcoholic fatty liver disease (NAFLD).

CONCLUSION
Metabolic stress-induced hepatocyte injury exhibited dual effects on autophagy and JZG activated the entire process, resulting in beneficial effects in NAFLD.

Key words: Autophagy; PI3K-AKT-mTOR signaling pathway; Autophagic flux; Oxidative stress; Hepatocyte injury; Non-alcoholic fatty liver disease

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Core tip: Non-alcoholic fatty liver disease (NAFLD), which is mainly characterized by the accumulation of lipids and energy metabolism dysfunction, is now one of the most common risk factors worldwide. As studies have demonstrated that autophagy is important in the maintenance of normal hepatocyte function and in the response to pathogenic changes, we examined the potential role of autophagy in metabolic stress-induced hepatocyte injury. The results showed that metabolic stress had dual effects on autophagy, resulting in autophagy induction and autophagic flux inhibition. The Chinese herbal formula Jiang Zhi Granule activated the autophagy process to protect against metabolic stress-induced hepatocyte injury in NAFLD.

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INTRODUCTION
Fatty liver disease (FLD), which is mainly characterized by the accumulation of lipids in hepatocytes and energy metabolism dysfunction, is clinically classified into two broad categories: alcoholic (A)FLD and non-alcoholic (NA)FLD[1]. NAFLD is currently much more prevalent than AFLD, which accounts for 75% of all chronic liver diseases, and is increasingly recognized as one of the most common risk factors worldwide[2]. NAFLD has a wide clinical pathological spectrum, ranging from simple steatosis non-alcoholic fatty liver to non-alcoholic steatohepatitis, which includes fibrosis and cirrhosis[3]. And, in the meantime, it often occurs with other metabolic diseases, such as obesity and diabetes[4].

The pathogenesis of NAFLD is still unknown; however, the “two hits” hypothesis is now widely accepted[5]. A disturbance in metabolic homeostasis is one of the key events during the occurrence of NAFLD and typical pathological features, including steatosis, inflammation, fibrosis and cirrhosis, are considered to be related to oxidative stress resulting from lipid accumulation and reactive oxygen species (ROS) generation[6].

Oxidative stress is a stimulus for autophagy, which is important for metabolic homeostasis in the liver[7], as it can prompt nutrient recycling, remove abnormal organelles and toxic protein aggregates and alter the level of metabolic factors, thus contributing to the maintenance of normal hepatocyte function and the response to pathogenic changes in the liver[8,9]. Hepatic autophagy occurs at a basal level and can be elevated under stress conditions[10], such as oxidative stress.

Studies have shown that autophagy is a highly selective process and can modulate cellular energy stores, such as carbohydrates and lipids[11,12], and recent research has demonstrated that autophagy assists in the degradation of triglycerides in the liver[13]. Autophagy regulators, such as rapamycin and carbamazepine, have been proven effective to improve hepatic function[14]. However, no effective therapeutic approach has been accepted as the standard option for NAFLD and its complications. Thus, new treatments are still urgently needed to prevent or delay the onset as well as the progression of NAFLD.

Jiang Zhi Granule (JZG), which is composed of Herba gynostemmatis, Radix salviae, Rhizoma polygoni cuspidati, Herba artemisiae scopariae and Folium nelumbinis, is a clinically used herbal formula designed to treat patients with NAFLD[15]. JZG had a positive drug safety evaluation and has been approved for clinical trials by the State Food and Drug Administration (SFDA; Authorization Number: Z10960082). Our preliminary studies indicated that JZG had beneficial effects in improving hepatic fat accumulation in both cell lines and animals[16], and the efficacy of JZG in patients with
NAFLD was also confirmed. As previous studies on JZG have indicated its antisteatotic effect, we conducted this study to determine the underlying mechanism of JZG.

MATERIALS AND METHODS

Animal models
Male C57BL/6 mice aged 6 wk were purchased from SLAC Animal Laboratories (Shanghai, China). After 1-wk acclimatization, the mice were divided into three groups: the control group (n = 10) received a 12-wk standard diet (STD); the high fat (HF) group (n = 13) received a HF diet (HFD; consisting of 10% lard oil, 2% cholesterol and 88% STD) supplemented with 1% DDS (MP Biomedicals, Solon, OH, United States) in drinking water; and, the JZG group (n = 13) also received a HF-DDS diet, but were also given JZG which had been dissolved in saline and was administered daily by oral gavage at a dose of 994 mg/kg daily (approximately 12 times that of the standard dose used in clinical practice). DSS was given in cycles; each cycle consisted of 7 d DSS administration followed by a 10-d interval with normal drinking water. At the end of the experimental period, blood samples were drawn from the heart, while the mice were under anesthesia, livers were excised, and samples were either immediately snap-frozen in liquid nitrogen or fixed in 4% PFA.

Cell culture
HepG2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Saturated palmitic acid (PA) and JZG were used in this study. HepG2 cells were incubated in culture medium containing PA (0, 0.1, 0.2, 0.3, 0.4 or 0.5 mmol/L) and treated with or without JZG (100 μg/mL) for 24 h or 48 h, as described previously.

Western blot
Western blot analyses were performed as described previously. Rabbit antibodies against LC3 (monoclonal, ab192890), SQSTM1/p62 (monoclonal, ab109012), p-mTOR (phosphor-S2481) (monoclonal, ab137133), mTOR (monoclonal, ab67540), p-PI3K (phosphor-Y607) (polyclonal, ab182651), PI3K (monoclonal, ab40755) and actin (polyclonal, ab8227) were purchased from Abcam (Cambridge, MA, United States).

Establishment of stable fluorescence-expressing cell lines
HepG2 cells were cultured according to the above protocols and infected with mRFP-GFP-LC3 lentivirus at an MOI of 10 for 48 h to establish a stable mRFP-GFP-LC3-expressing HepG2 cell line, and then infected with mCherry-p62 lentivirus at an MOI of 10 for 48 h to establish a stable mCherry-p62-expressing HepG2 cell line.

RESULTS

Autophagy was activated by JZG to protect against injury in PA-treated cells
To determine whether autophagy was involved in the progression of NAFLD, HepG2 cells were treated with PA - a main type of saturated free fatty acid which is elevated in obese subjects and can induce NAFLD - at various concentrations and for different time periods. Results showed that the expression of alanine aminotransferase (ALT) increased in a dose- and time-dependent manner and JZG significantly reduced high ALT levels (Figure 1A and B), suggesting that cell trauma was induced by PA and JZG improved hepatic function. These results also indicated that the dose of 0.4 mmol/L may be an appropriate concentration in subsequent experiments and the time points should include 24 h and 48 h. Western blot analyses showed that PA increased LC3-Ⅱ/actin expression (Figure 1C), indicating the activation of autophagy. However, the expression of LC3-Ⅱ/LC3-Ⅰ was not increased accordingly after long-term PA stimulation (Figure 1C). In the meantime, the expression of p62 was also increased (Figure 1C), suggesting the restriction of autophagic flux. Thus, it was proposed that PA exhibited dual effects on...
An increase in LC3-II/LC3-I expression and a reduction in p62 expression were observed following treatment with JZG for two different time periods (Figure 1D and E), indicating that JZG activated autophagy to protect against PA-induced cell injury.

**PI3K-AKT-mTOR pathway was involved in autophagy in JZG-treated cells**

As the activation of autophagy can occur due to an

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**Figure 1** Autophagy was activated by JZG to protect against injury in palmitate-treated cells. A: The expression of ALT in the supernatants of cell cultures was determined by ELISA. HepG2 cells were incubated in culture medium containing PA (0, 0.1, 0.2, 0.3, 0.4 or 0.5 mmol/L); B: The expression of ALT in the supernatants of cell cultures was determined by ELISA. HepG2 cells were treated with 0.4 mmol/L PA with or without JZG (100 μg/mL) for different time periods (0, 6, 12, 24 or 48 h); C: HepG2 cells were treated with 0.4 mmol/L PA for different time periods (0, 12, 24 or 48 h); D: HepG2 cells were treated with 0.4 mmol/L PA and rapamycin (2 μmol/L) or JZG (100 μg/mL) for 24 h; E: HepG2 cells were treated with 0.4 mmol/L PA and rapamycin (2 μmol/L) or JZG (100 μg/mL) for 48 h. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. ALT: Alanine aminotransferase; ELISA: Enzyme-linked immunosorbent assay; JZG: Jiang Zhi Granule; PA: Palmitate.
increase in autophagy induction and autophagic flux, we first investigated the pathway involved in autophagy induction in JZG-treated cells. The PI3K-AKT-mTOR pathway, a classic signaling pathway that has been identified to be important in autophagy induction, was examined in this study. Western blot analyses revealed that the phosphorylation signaling processes of mTOR and PI3K were inhibited in PA-treated cells, and these results were further confirmed by the addition of rapamycin (Figure 2A and B). As an inhibitor of mTOR, rapamycin did not further depress the phosphorylation of PI3K. Conversely, an additional restraint on both phosphorylation signaling processes was observed in JZG-treated cells (Figure 2A and B), suggesting that
the PI3K-AKT-mTOR pathway is critically involved in autophagy induction in response to PA challenge.

As LC3 is the major constituent of autophagosomes, a stable mRFP-GFP-LC3-expressing HepG2 cell line was established to visualize the progression of autophagosome formation in real time in liver cells. Diffuse cytoplasmic localization of mRFP-GFP-LC3 was observed in the control group, whereas punctate fluorescence was observed in PA-treated cells (Figure 2C), indicating that cytoplasmic LC3 was processed and recruited to autophagosomes. Rapamycin and JZG advanced this process, indicating increased autophagy induction, which confirmed that JZG induced the formation of autophagosomes.

Figure 3 Effects of Jiang Zhi Granule on autophagic flux in palmitate-treated cells. A: HepG2 cells stably expressing mCherry-p62 were pretreated with 0.4 mmol/L PA and rapamycin (2 μmol/L) or JZG (100 μg/mL) for 24 h and 48 h, and then analyzed by fluorescence microscopy; B: HepG2 cells were treated with 0.4 mmol/L PA and rapamycin (2 μmol/L) or JZG (100 μg/mL) for 48 h, and then analyzed by fluorescence microscopy. JZG: Jiang Zhi Granule; PA: Palmitate.
Effects of JZG on autophagic flux in PA-treated cells
To determine the effect of JZG on autophagic flux in PA-treated cells, we first observed this effect in mRFP-GFP-LC3-expressing HepG2 cells. We found that yellow fluorescence - indicating that GFP was not degraded by lysosomal enzymes - was largely seen in HepG2 cells treated with PA for 48 h, whereas red fluorescence was very rare (Figure 2C). These results led us to propose that the colocalization of autophagosomes and lysosomes was prevented and the autophagosomal degradation was blocked. Yellow punctate fluorescence was reduced in JZG-treated cells and red diffuse fluorescence was increased (Figure 2C), suggesting that JZG promoted colocalization.

To confirm these findings, we established another stable mCherry-p62-expressing HepG2 cell line to visualize the cellular expression levels of p62, which inversely correlated with autophagic flux via selective incorporation into autophagosomes to be efficiently degraded by autophagy. We found that punctate fluorescence was enhanced in HepG2 cells treated with PA for 48 h and was weak in JZG-treated cells (Figure 2C), confirming the previous findings. As it has been shown that mitochondrial dysfunction may block autophagic flux\(^{1,22}\), we next examined whether the mitochondrial integrity was affected in PA-treated cells. MitoTracker and LysoTracker were used to stain the mitochondria and lysosomes, respectively. The results showed that mitochondrial dysfunction was much more serious in PA-treated cells than in control cells (Figure 3B). A protective effect of JZG on mitochondrial integrity was demonstrated and the colocalization of mitochondria and lysosomes in JZG-treated cells showed that the mitochondrial integrity was related to activation of autophagic flux. Together, these findings suggested that JZG increased the formation of both autophagosomes and autolysosomes and protected against PA-induced mitochondrial dysfunction by activating autophagy.

\textbf{JZG protected mitochondrial integrity against oxidative stress}
As the protective effect of JZG on mitochondrial integrity was demonstrated, we then examined whether oxidative stress was involved in this process. The ROS-sensitive fluorescent probe DCFH-DA was used to monitor mitochondrial membrane potential in HepG2 cells pretreated with 0.4 mmol/L PA and rapamycin (2 \(\mu\)mol/L) or JZG (100 \(\mu\)g/mL) for 24 h and 48 h, and the cells were then analyzed by fluorescence microscopy (Figure 4A). JC-1 was used to monitor mitochondrial membrane potential in HepG2 cells pretreated with 0.4 mmol/L PA and rapamycin (2 \(\mu\)mol/L) or JZG (100 \(\mu\)g/mL) for 24 h and 48 h, and the cells were then analyzed by fluorescence microscopy (Figure 4B). JZG: Jiang Zhi Granule; PA: Palmitate; ROS: Reactive oxygen species.

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\caption{Jiang Zhi Granule protected mitochondrial integrity against oxidative stress. A: The ROS-sensitive fluorescent probe DCFH-DA was used to monitor mitochondrial membrane potential in HepG2 cells pretreated with 0.4 mmol/L PA and rapamycin (2 \(\mu\)mol/L) or JZG (100 \(\mu\)g/mL) for 24 h and 48 h, and the cells were then analyzed by fluorescence microscopy; B: JC-1 was used to monitor mitochondrial membrane potential in HepG2 cells pretreated with 0.4 mmol/L PA and rapamycin (2 \(\mu\)mol/L) or JZG (100 \(\mu\)g/mL) for 24 h and 48 h, and the cells were then analyzed by fluorescence microscopy. JZG: Jiang Zhi Granule; PA: Palmitate; ROS: Reactive oxygen species.}
\end{figure}
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(Figure 4A), indicating the potential role of oxidative stress in mitochondrial dysfunction. We also monitored mitochondrial membrane potential with JC-1 to evaluate oxidative damage, and the results revealed a reduction in mitochondrial membrane potential in PA-treated cells, and JZG prevented this PA-induced reduction (Figure 4B), confirming that JZG protected mitochondrial integrity against oxidative stress in PA-induced mitochondrial dysfunction.

**Autophagy was activated by JZG to improve NAFLD in vivo**

A murine model of NAFLD induced by HFD was employed to assess the potential role of autophagy in metabolic stress-induced liver injury and inflammation. As expected, the HFD increased both the expression of LC3-II/actin and p62 (Figure 5A), suggesting the activation of autophagy induction and inhibition of autophagic flux. JZG treatment induced an increase in LC3-II/LC3-I expression and led to a decrease in p62 expression (Figure 5A), indicating up-regulation of the autophagy pathway in JZG-treated mice.

Biochemical analyses showed that HFD elevated the expression of circulating ALT, aspartate aminotransferase, total cholesterol, triglycerides and fasting blood glucose as well as hepatic triglycerides, and JZG improved the metabolic disorder and associated liver injury (Figure 5B). The results of HE and oil red O staining demonstrated that lipid droplets and inflammation were induced by HFD and JZG ameliorated these conditions (Figure 5C). Together, these findings suggested that JZG had beneficial effects in improving NAFLD in vivo.

**DISCUSSION**

NAFLD, as the leading cause of chronic liver disease, could result in serious liver-related complications and an increase in overall mortality. In previous research, we demonstrated that the Chinese herbal formula, JZG, had beneficial effects in improving hepatic fat accumulation, and in this study, we showed that autophagy is critically involved in this process.

Autophagy occurs when autophagosomes are formed and autophagy induction is attributed to various origins, such as the endoplasmic reticulum, the Golgi apparatus, the mitochondria or the plasma membrane[23,24]. The autophagosomes then become autolysosomes by fusing with lysosomes and degrading the components in cytosols[25,26]. Thus, the upstream event of autophagy induction was presented by LC3-II/actin expression in this study, and the downstream event of autophagic flux was presented by the expression of LC3-II/LC3-I. Stable fluorescence-expressing cell lines of LC3 were established to visualize the whole progression of autophagic flux. Results showed that metabolic stress-induced hepatocyte injury exhibited dual effects on autophagy by activating autophagy induction and blocking autophagic flux.

A series of signaling pathways and regulators which regulate autophagy have been identified in the past decade. In this research, a classic signaling pathway, the PI3K-AKT-mTOR signaling pathway[27], was confirmed to be important in response to PA challenge. As the...
core target in this signaling pathway, mTOR, a master regulator of cellular metabolism, can be stimulated by multiple stimulants, such as nutritional status, hormonal factors and oxygen concentrations\(^2\). Under these conditions, mTOR complex 1 (mTORC1) will inhibit the ULK complex by phosphorylating Atg13 and ULK1/2, which results in autophagy suppression\(^2\). However, restraints on both phosphorylation signaling processes of PI3K and mTOR were observed in JZG-treated cells, indicating that the PI3K-AKT-mTOR pathway was involved in autophagy in the JZG-treated cells.

Subsequent studies have shown that SQSTM1/p62 accumulation is correlated with NAFLD and the fusion of isolated hepatic autophagosomes and lysosomes is different in NAFLD patients\(^3\), which suggests that an excessive amount of lipids may contribute to SQSTM1/p62 accumulation by suppressing autophagosomes/lysosome fusion\(^3,4\). Thus, we examined the SQSTM1/p62 accumulation and the results confirmed previous findings.

Fluorescence staining was performed to connect the mitochondrial integrity with oxidative stress induced by PA. We also found that JZG could activate the autophagy process by either induction of autophagosomes or colocalization of autophagosomes and lysosomes as well as degradation of autolysosomes to protect against metabolic stress-induced hepatocyte injury in NAFLD (Figure 6).

Limitations should be acknowledged. The complex compounds contained in this prescription may have led to multitarget effects, and a series of signaling pathways and regulators were not examined. Beyond that, as p62 could also be degraded by proteasome, further studies on this aspect should be continually conducted to confirm the findings in the future. However, as the current epidemic of obesity and obesity-related NAFLD continues to increase, new approaches for prevention and treatment are urgently needed, and traditional Chinese medicine, as an alternative and complementary medicine, may be an effective addition to the current standardized intervention strategy.

ARTICLE HIGHLIGHTS

Research background
Non-alcoholic fatty liver disease (NAFLD), as the leading cause of chronic liver disease, can result in serious liver-related complications and an increase in overall mortality. However, the pathogenesis of NAFLD is still unknown and no effective therapeutic strategy has been accepted as the standard treatment option. In previous studies, the authors found that JZG had beneficial effects in improving hepatic fat accumulation, metabolic disorder and associated liver injury, and its efficacy in patients with NAFLD was also confirmed.

Research motivation
Autophagy is important in liver diseases, and research has demonstrated that autophagy regulators can improve hepatic function. However, no effective therapeutic strategy has been accepted as the standard option for NAFLD and
its complications. Thus, novel treatments are still urgently needed to prevent or delay the onset as well as the progression of NAFLD.

Research objectives
NAFLD, as the leading cause of chronic liver disease, could result in serious liver-related complications and an increase in overall mortality. And traditional Chinese medicine, as an alternative and complementary medicine, may be an effective addition to the current standardized intervention strategy.

Research methods
The process of autophagy was detected by the expressions of LC3 and SQSTM1/p62. Stable fluorescence-expressing cell lines were established with mRFP-GFP-LC3 and mCherry-p62 lentivirus to visualize the whole progression of autophagic flux.

Research results
In previous research, the authors had demonstrated that the Chinese herbal formula JZG had beneficial effects in improving hepatic fat accumulation. In this study, autophagy was demonstrated to be critically involved in this process.

Research conclusions
The authors confirmed that metabolic stress-induced hepatocyte injury exhibited dual effects on autophagy, while JZG activated the whole process to provide beneficial effects in NAFLD.

Research perspectives
The exact compounds contained in this prescription are still unknown and the complex compounds might have led to multitarget effects. A systems pharmacology approach to determine the active compounds and action mechanisms might be a good method for the future research.

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