

# Endoplasmic reticulum stress induced by lipopolysaccharide is involved in the association between inflammation and autophagy in INS-1 cells

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Received October 18, 2016; Accepted June 13, 2017

DOI: 10.3892/mmr.2017.7350

**Abstract.** Type 2 diabetes is a chronic inflammatory disease. Autophagy, the dynamic process of lysosomal degradation of damaged organelles and proteins, may protect  $\beta$ -cells from destruction by inflammation in type 2 diabetes. The present study investigated the role of autophagy, inflammation and endoplasmic reticulum (ER) stress in type 2 diabetes. INS-1 cells were incubated with lipopolysaccharide. The chemical chaperone 4-phenylbutyric acid was used to inhibit ER stress, and 3-methyladenine (3-MA) was used to inhibit autophagy. Apoptosis was detected by flow cytometry and cell proliferation using Cell Counting kit-8 solution. Light chain-3B, interleukin (IL) 1 $\beta$ , caspase-1 and C/EBP homologous protein production were assessed by western blotting, and rat activating transcription factor 4 and rat binding immunoglobulin heavy chain protein gene expression were determined by real-time reverse transcription-polymerase chain reaction. The results showed that inhibiting autophagy with 3-MA unexpectedly contributed to cell death in  $\beta$ -cells. This response was associated with an increase in inflammatory cytokines, including IL1 $\beta$  and caspase-1. Inhibiting ER stress with 4-phenylbutyric acid led to a decrease in cell apoptosis. These results showed that autophagy may have a protective effect by reducing inflammatory cytokines in  $\beta$ -cells. In addition, the inositol-requiring enzyme 1 pathway mediated the ER stress associated with autophagy and inflammatory cytokines (IL1 $\beta$  and caspase-1). Therefore, inflammatory cytokines may be critical signalling nodes, which are associated with ER stress-mediated  $\beta$ -cell death.

## Introduction

Inflammation has been implicated in the pathophysiology of type 2 diabetes. During insulinitis, activated macrophages and T-cells release cytokines, including interleukin (IL) 1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$ , close to  $\beta$ -cells, contributing to  $\beta$ -cell dysfunction and death (1,2). Additionally, there is evidence that elevated levels of IL1 $\beta$ , IL6, monocyte chemoattractant protein 1 and C-reactive protein are predictive of type 2 diabetes (3). Previous findings suggest that islet  $\beta$ -cells secrete cytokines by themselves, which induce inflammatory responses. In this process, thioredoxin-interacting protein (TXNIP) is induced by endoplasmic reticulum (ER) stress through the protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1 (IRE1) pathways, which activates the production of IL1 $\beta$  by the NOD-like receptor (NLRP) 3/caspase1 inflammasome, and mediates ER stress-mediated  $\beta$ -cell death (4).

Autophagy is a major pathway for the delivery of proteins, organelles, lipids, DNA and RNA to lysosomes, where they are to be degraded and recycled in the vacuole. Autophagy provides pools of raw material for anabolic processes and drives a continuous flow of materials in a degradation-regeneration cycle within the cell (5). Paradoxically, autophagy is considered to be a cell-fate decision maker and may lead to a form of non-apoptotic cell death, which is termed type 2 programmed cell death (6). Therefore, whether autophagy protects cells from diverse types of injuries or promotes cell death may depend on either the cellular or the environmental context.

At present, it is known that autophagy is induced by ER stress in mammalian cells and in plants (7,8). Although the molecular mechanism linking ER stress and autophagy remains to be elucidated, the process of cleavage and lipidation of microtubule-associated protein 1 light chain 3 (LC3) into LC3-II is reportedly mediated by the phosphorylation of PERK/eukaryotic translation initiation factor 2A (eIF2a) (6). No autophagosomes were observed in an *ire1 $\beta$* -knockout mutant when treated with ER stress agents, suggesting that the ER stress sensor, IRE1 $\beta$  is required for ER stress-induced autophagy (8,9).

All of the above factors may be involved in diabetes. In the present study, the potential involvement of autophagy in the inflammatory response was investigated in INS-1 cells.

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**Abbreviations:** 3-MA, 3-methyladenine; ER, endoplasmic reticulum; PBA, 4-phenylbutyric acid; NLRP, NOD-like receptor; LC3, light chain 3; LPS, lipopolysaccharide; CCK8, Cell Counting kit-8; AV, autophagic vacuoles; AL, autophagolysosomes

**Key words:** type 2 diabetes, autophagy, inflammatory cytokines, endoplasmic reticulum stress, lipopolysaccharide

In addition, whether IRE-1-pathway-mediated ER stress is involved in the associated between inflammation and autophagy in diabetes was examined.

## Materials and methods

**Cell culture.** INS-1 cells from rats (cells were provided by the Department of Endocrinology, The Second Affiliated Hospital of Harbin Medical University, Heilongjiang, China) were passaged in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Sijiqing, Hangzhou, China), in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> (10). The cells were treated with 100 ng/ml lipopolysaccharide (LPS) to activate ER stress-induced inflammatory cytokines, including IL1 $\beta$  and caspase-1. 4-phenylbutyric acid (PBA; 2.5 mmol/l; pH 7.4; Sigma; Merck Millipore, Darmstadt, Germany) was used to inhibit the IRE-1 pathway and mediate ER stress, and 3-methyadenine (3-MA; 5 mmol/l; Sigma; Merck Millipore) was used to inhibit autophagy (10,11).

**Cell Counting kit-8 (CCK8) viability assay.** The viability of cells was assessed using CCK8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. CCK8 is more sensitive than 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (10). The INS-1 cells were plated in 96-well plates at a density of  $5 \times 10^7 l^{-1}$  and treated with 10 ml CCK8 at 37°C for 1 h. Absorbance was measured at 450 nm using a microplate reader (10).

**Annexin V/propidium iodide (PI) staining.** Cell death was determined using flow cytometry. The INS-1 cells were stained with Annexin V and PI using the Annexin-V-FLUOS staining kit (Roche Diagnostics, Basel, Switzerland) in accordance with the manufacturer's protocol. Briefly, the cells were plated in 24-well plates at a density of  $1.2 \times 10^5$  cells per cm<sup>2</sup>, treated with lysosomal proteases and stained with an incubation buffer containing Annexin V and PI for 10 min at 15–25°C in the dark. Images of the apoptotic cells were captured using a fluorescence microscope (Nikon Eclipse TE2000-U; Nikon Corporation, Tokyo, Japan) with an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (12).

**Western blot analysis.** Cells were washed with PBS and lysed in lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% BME, 1% TritonX-100, 1 mM EDTA, 1 mM EGTA, 10 mM DTT and 1 mM Na<sub>3</sub>VO<sub>4</sub>]. The lysates were then incubated on ice for 30 min and centrifuged at 8,000 x g for 5 min at 4°C. Protein concentrations were determined using the Bicinchoninic Acid method. Equal quantities of protein (50  $\mu$ g) were resolved by 10–15% SDS-PAGE, transferred onto polyvinylidene difluoride membranes and blocked with 5% skim milk at room temperature for 1 h. The membranes were incubated with specific primary antibodies overnight at 4°C. The following antibodies were used at a 1:1,000 dilution (unless otherwise indicated): LC3 (Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 2775), IL1 $\beta$  (Cell Signaling Technology, Inc.; cat. no. 2002), caspase-1 (Abcam, Cambridge, UK; cat. no. 2225), C/EBP homologous protein (CHOP; Cell Signaling

Technology, Inc.; cat. no. 2895) and  $\beta$ -actin (Cell Signaling Technology, Inc.; cat. no. 8475). The membranes were washed with PBS-0.1% Tween-20, then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; Sigma; Merck Millipore) at room temperature for 1 h (10). Protein bands were detected using Enhanced chemiluminescence (Beyotime Institute of Biochemistry, Haimen, China). Immunoblots were quantified by densitometric analysis using Image Lab software v2.0.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The quantification of protein phosphorylation was normalized to the corresponding total protein expression. The relative expression level of a certain protein was normalized to  $\beta$ -actin and experiments were repeated in triplicate.

**RNA analysis.** To analyze mRNA expression, total RNA was extracted using TRNzol reagent (Tiangen Biotech Co., Ltd., Beijing, China; cat. no. DP405-02) according to the manufacturer's instructions. The purity and integrity of the RNA were examined spectroscopically prior to obtaining cDNA using the Nanodrop 2000. Reverse transcription was performed using the PrimeScript™ RT reagent kit with gDNA Eraser system (Takara Bio, Inc., Otsu, Japan; cat. no. RR047B) according to the manufacturer's instructions. The reactions were set up with the following master mix: 2.0  $\mu$ l gDNA Eraser Buffer, 1.0  $\mu$ l gDNA Eraser, 1  $\mu$ g Total RNA and 6.0  $\mu$ l RNase Free H<sub>2</sub>O, which was incubated for 2 min at 42°C. This master mix was then mixed with 1.0  $\mu$ l PrimeScript RT Enzyme Mix I, 1.0  $\mu$ l RT Primer Mix, 4.0  $\mu$ l PrimeScript Buffer 2 and 4.0  $\mu$ l RNase Free H<sub>2</sub>O, and incubated at 37°C for 15 min then at 85°C for 5 sec. For thermocycling reactions, the ABI prism 7500 sequencer detection system was used. Using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) and the ROX plus reaction system (both Takara Bio, Inc.; cat. no. RR82LR), the following thermocycling conditions were applied: 95°C for 30 sec, then 45 cycles of 95°C for 5 sec and 60°C for 40 sec. The relative quantity of each transcript was calculated by a standard curve of quantification cycle values for serial dilutions of a cDNA sample. PCR was performed in triplicate for each sample. The following sets of primers were used for PCR analysis: Rat actin forward 5'-GGA GATTACTGCCCTGGCTCCTA-3' and reverse 5'-GACTCA TCGTACTCCTGCTTGCTG-3'; rat binding immunoglobulin heavy chain protein (Bip), forward 5'-GAATCCCTCCTGCTC CCCGT-3' and reverse 5'-TTGGTCATTGGTGATGGTGAT TTTG-3'; rat activating transcription factor 4 (ATF4), forward 5'-TATGAGCCCTGAGTCCTACCTG-3' and 5'-CTGCTG TCTTGTTTTGCTCCAT-3'. The primers used for analysis were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All experiments were performed in triplicate and the relative mRNA levels were analyzed using the 2<sup>- $\Delta\Delta$ Cq</sup> method (13).

**Statistical analysis.** The results are expressed as the mean  $\pm$  standard error of the mean. Comparisons of a single variable in >2 groups were analyzed using one-way analysis of variance followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed using the paired and unpaired t-test between two groups using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

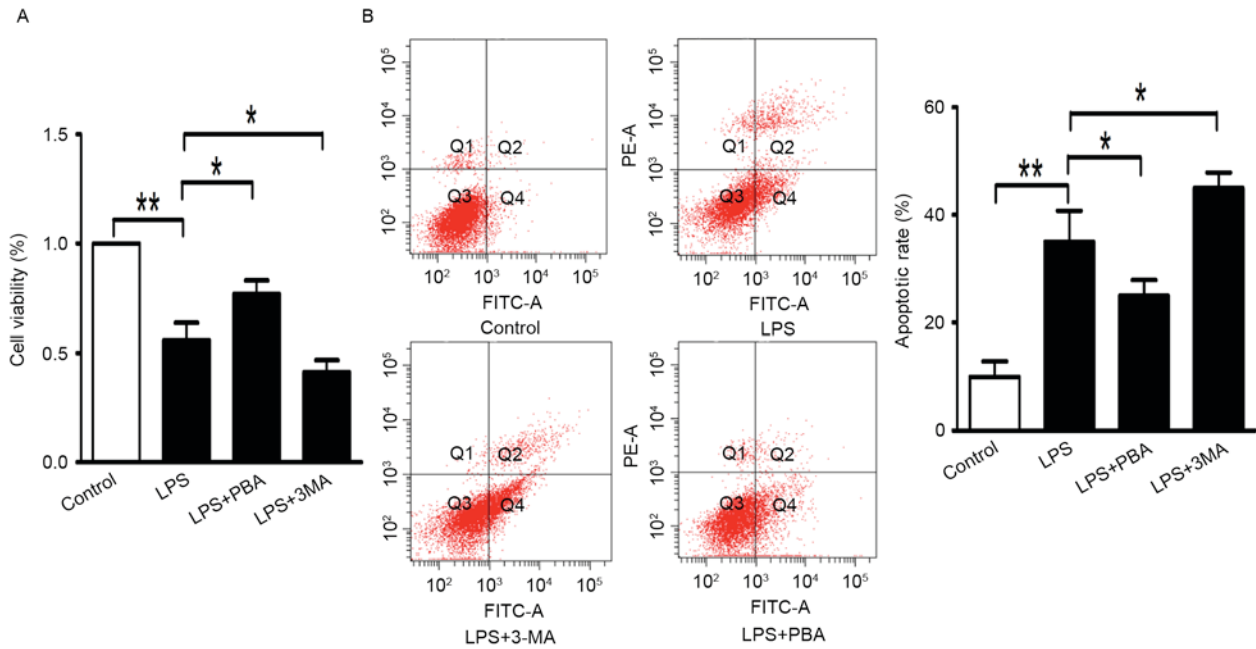


Figure 1. Detection of the survival status of INS-1 cells treated with LPS and inhibitor of endoplasmic reticulum stress/autophagy. INS-1 cells were incubated with LPS 100 ng/ml for 24 h to simulate the conditions of diabetes. INS-1 cells were treated with a combination of LPS and PBA (5 mmol/l)/3-MA (5 mmol/l) for 24 h as the LPS+PBA group and the LPS+3-MA group, respectively. (A) Relative cell viability was determined using a Cell Counting kit-8 assay. (B) Apoptotic rates were determined using flow cytometry. Values are presented as the mean  $\pm$  standard deviation (n=3). \*P<0.05, \*\*P<0.01. LPS, lipopolysaccharide; PBA, 4-phenylbutyric acid; 3-MA, 3-methyadenine.

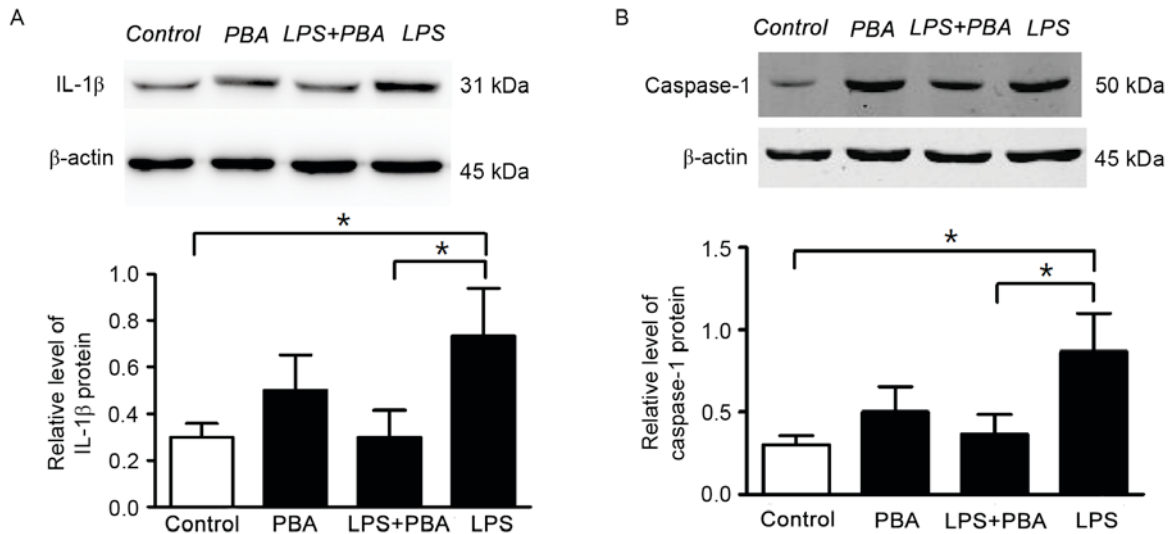


Figure 2. Detection of protein expression levels of mature caspase-1 and inflammatory cytokines IL-1 $\beta$  in INS-1 cells treated them LPS or LPS and the inhibitor of endoplasmic reticulum stress. (A) Protein levels of IL-1 $\beta$  and (B) caspase-1 were normalized to  $\beta$ -actin. Values are presented as the mean  $\pm$  standard deviation (n=3). \*P<0.05. LPS, lipopolysaccharide; PBA, 4-phenylbutyric acid; IL-1 $\beta$ , interleukin-1 $\beta$ .

## Results

**LPS induces the production of IL1 $\beta$ , inflammasome activation and apoptosis in INS-1 cells.** The INS-1 cells were incubated with 100 ng/ml LPS in RPMI-1640 for 24 h. As shown in Fig. 1A, the viability of the INS-1 cells analyzed using the CCK8 assay was reduced to 64% with LPS treatment, compared with that in the control group. Apoptosis of cells in the LPS group was increased by 26.8%, determined using an Annexin V-FITC/PI quantification assay (Fig. 1B).

**PBA attenuates LPS-induced production of IL1 $\beta$  and enhances survival of in INS-1 cells.** The INS-1 cells were treated with LPS and PBA. PBA is a low-molecular-weight compound, which is known to reduce the load of unfolded proteins in the ER by facilitating folding capacity and trafficking of mutant proteins out of the ER. Ozcan *et al* (14) and Tang *et al* (15) demonstrated that PBA decreases the activation of IRE-1, but not of the PERK or ATF6 pathways. On the basis of previous experimental analysis, the present study used PBA as a chemical chaperone to inhibit IRE-1 pathway-mediated

ER stress. The survival of INS-1 cells was improved to 78% (Fig. 1A), and the percentage of apoptotic cells was reduced to 21.2% (Fig. 1B) following treatment with LPS and PBA (5 mmol/l) for 24 h. In the LPS+PBA-treated INS-1 cells, the protein expression levels of IL-1 $\beta$  (Fig. 2A) and caspase-1 (Fig. 2B) were reduced, compared with those in the groups treated with LPS alone. These results suggested that the modulation of ER stress reduced the production of inflammatory cytokine (IL-1 $\beta$ ) and improved INS-1 cell survival.

*IRE-1-pathway-mediated ER stress is involved in the process of inflammation and autophagy.* As LC3B-II is a key protein associated with autophagy, the conversion of LC3B-I to LC3B-II was examined in the present study. There were fewer autophagic vacuoles (AVs; Fig. 3A, blue arrowheads), and a number of swelling mitochondria (Fig. 3A, green arrowheads) detected in the LPS+PBA group, determined using EM. In addition, the LPS-induced LC3B-II protein levels were reduced in the LPS+PBA group, compared with those in the LPS-treated group (Fig. 3B). These results suggested that IRE-1-pathway-mediated ER stress was involved in the association between inflammation and autophagy, and in promoting the processes of inflammation and autophagy.

*Autophagy decreases INS-1 cell death induced by the inflammatory response.* To determine the role of autophagy in the inflammatory response, the INS-1 cells were treated with autophagy inhibitor (3-MA). INS-1 cell viability was significantly reduced to 42% by treatment with LPS+3-MA (Fig. 1A). 3-MA increased the apoptotic rate to 43.8% in the LPS+3-MA group (Fig. 1B). Additionally, the typical autophagosome (Fig. 3A, red arrowhead) double-limiting membrane in the LPS-treated INS-1 cells was detected by EM. Double-membrane autophagic vesicles containing cell organelles in the cytoplasm of INS-1 cells is an integrated autophagosome, shown by ultrastructural image analysis. There were numerous different periods of autophagosomes (Fig. 3A, red arrowheads) and autophagolysosomes (ALs) in the LPS group. These results indicated that autophagy had a protective effect on the LPS-induced inflammatory response in INS-1 cells and was necessary to maintain the normal architecture and function of INS-1 cells. The mRNA expression levels of Bip and ATF4, and the protein expression of CHOP were assessed following treatment with 3-MA. The mRNA levels of Bip and ATF4, and the protein expression of CHOP were modestly upregulated (Fig. 4A-C). These data suggested that autophagy protected INS-1 cells by attenuating excessive ER stress, and decreasing cell apoptosis mediated by PERK/CHOP-mediated ER-stress and the production of inflammatory cytokines (IL1 $\beta$ ).

## Discussion

Type 2 diabetes is closely associated with the activation of inflammatory signaling pathways, resulting in a marked increase in autophagy, in addition to abnormal cytokine production (16). These results suggested that pancreatic  $\beta$  cells can secrete cytokine IL1 $\beta$ , which is involved in the inflammatory response and is detrimental to cell survival. Autophagy is also involved in type 2 diabetes, however,

whether autophagy promotes INS-1 cell death or protects cells from injuries remains to be elucidated. Therefore, INS-1 cells were treated with autophagy inhibitor (3-MA). The further decrease in proliferation and increased apoptosis of INS-1 cells suggested that autophagy protected the cells from death.

Type 2 diabetes is associated with insulin resistance, cell depletion, and immune attack, involving lipotoxicity, glucotoxicity and inflammation (17). Several studies have demonstrated that the intra-islet expression of inflammatory cytokines, particularly IL-1 $\beta$ , contributes to the pathogenesis of type 2 diabetes. The most prominent feature of type 2 diabetes, compared with type 1 diabetes, is that the islet inflammatory response is 'low-grade' and its role in the pathophysiology of type 2 diabetes is somewhat controversial (18). In the present study, pancreatic  $\beta$  cells secreted cytokines, including IL1 $\beta$ , which was involved in the inflammatory response and is detrimental to the survival of  $\beta$ -cells. These results are consistent with a previous study by Böni-Schnetzler *et al* (19). This previous study differed in that the RNA from  $\beta$ -cells of individuals with type 2 diabetes were analyzed, and it was revealed that the mRNA expression of IL1 $\beta$  was induced by high glucose and IL1 $\beta$  autostimulation, and was decreased by the IL-1 receptor antagonist IL-1Ra. Therefore, the production of inflammatory cytokines may be a positive feedback mechanism, and the autostimulation of IL1 $\beta$  is transient and nuclear factor- $\kappa$ B-dependent (19).

Clinical and experimental evidence suggests that ER stress is a potent, evolutionarily conserved response to misfolded proteins and cellular metabolic stress, which contributes to the life-and-death decisions of  $\beta$ -cells during type 2 diabetes. The inflammatory response is frequently triggered as a consequence of ER stress, caused either by metabolic problems or by the accumulation of misfolded proteins (20). Osowski *et al* (4) showed that TXNIP is a critical signaling node, which links ER stress to inflammation. TXNIP induced by ER stress is under the control of PERK and IRE1 pathways, which induce the mRNA transcription of IL1 $\beta$  (4). IL1 $\beta$  is activated by the NLRP3 inflammasome and mediates ER stress-mediated  $\beta$ -cell death. Activation induces oligomerisation of the NLRP3 inflammasome and recruits ASC through a homotypic PYD-PYD interaction. The ASC then recruits pro-caspase-1, leading to the autocatalytic activation of caspase-1, and these active caspase-1 hetero-tetramers can convert inactive pro-IL1 $\beta$  into their bioactive and secreted forms (3). The results of the present study demonstrated that modulating ER and increase folding capacity can reduce inflammatory cytokines (IL1 $\beta$ ) and improve INS-1 cell survival. The results also suggested that IRE-1-pathway-mediated ER stress is a key molecule linking inflammation to autophagy, which may contribute to the death and dysfunction of  $\beta$ -cells, caused by a combination of glucotoxicity and lipotoxicity (21).

Autophagy is an evolutionary conserved process, which has two effects on cell survival. The data presented in the present study suggested that autophagy is a cell-protection mechanism. However, Eskelinen and Saftig suggested that, when the disposal mechanism is carried to excess, the cells are committed to die, dependent on autophagy (22). In mammalian cells, autophagy is activated by ER stress, with IRE1-JNK as a link between the two processes, and the kinase function

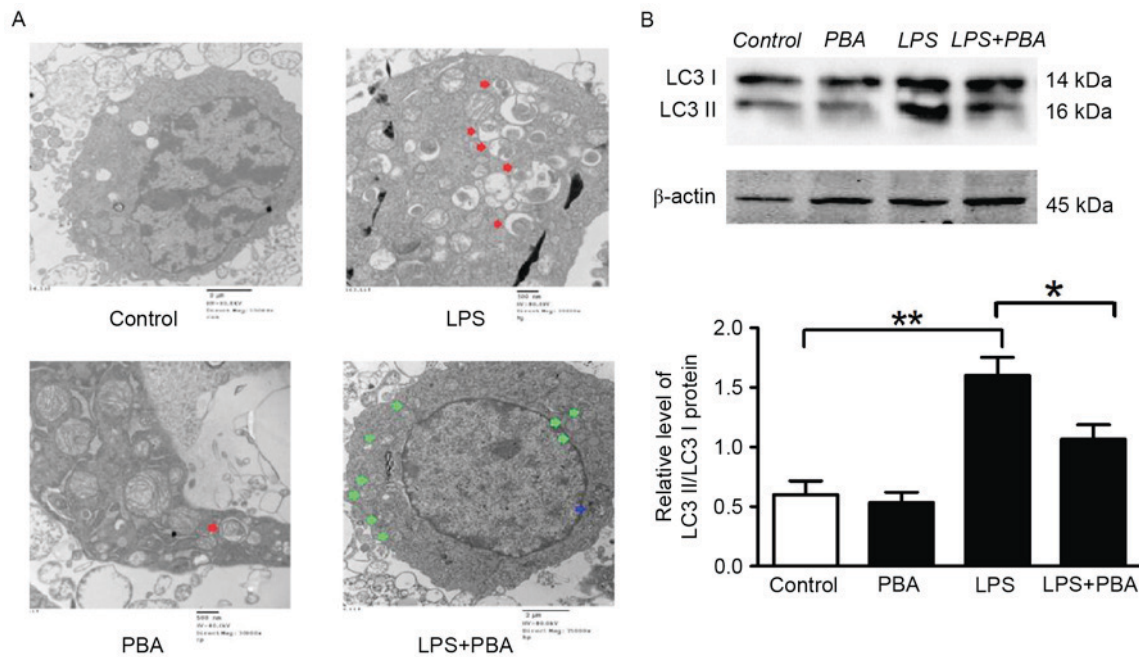


Figure 3. (A) Micromorphological changes in cellular organelles examined by transmission electron microscopy; autophagosomes (red arrowheads), autophagic vacuoles (blue arrowheads) and swelling mitochondria (green arrowheads) were observed. (B) Western blot analysis was used to detect the expression of LC3 in INS-1 cells. Relative expression of LC3 was normalized to  $\beta$ -actin. Data are expressed as the mean  $\pm$  standard deviation (n=3 for each group). \* $P$ <0.05, \*\* $P$ <0.01. LPS, lipopolysaccharide; PBA, 4-phenylbutyric acid; LC3, light chain 3.

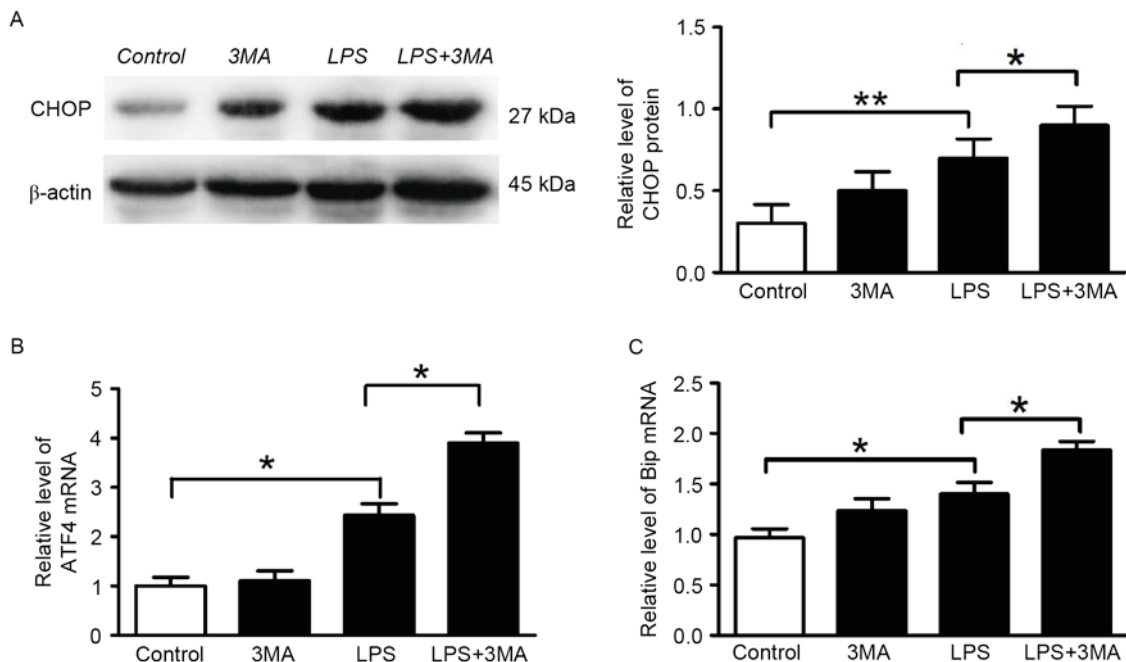


Figure 4. (A) Protein expression of CHOP; (B) mRNA expression of ATF4; (C) mRNA expression of Bip. Data are expressed as the mean  $\pm$  standard error of the mean (n=3 for each group). \* $P$ <0.05, \*\* $P$ <0.01, compared with the control. LPS, lipopolysaccharide; PBA, 4-phenylbutyric acid; 3-MA, 3-methyladenine; CHOP, C/EBP homologous protein; ATF4; activating transcription factor 4; Bip, binding immunoglobulin heavy chain protein.

of IRE1 is required for the induction of autophagy. However, PERK and ATF6 pathways are not considered to be important in the activation of autophagy following ER stress (7). In the present study, inhibiting IRE-1 mediated ER stress not only through reducing the protein expression of caspase-1 and IL1 $\beta$ , but also by depressing the LC3-II/LC3-I ratio, compared with levels in the LPS-treated group. There are two possible reasons

for these results. It may be that autophagy, which is induced by inflammatory cytokines, is reduced, followed by decreased expression levels of caspase-1 and IL1 $\beta$ . Alternatively, it may be that the autophagy induced by IRE-1-mediated ER stress is decreased. However, the production of inflammatory cytokines (IL1 $\beta$ ) is also mediated by the IRE-1 pathway. Therefore, the results of the present study suggested that IRE1 is a link

between autophagy and ER stress in INS-1 cells. Additionally, Gonzalez *et al* (6) suggested that LC3I can be converted to the lipidated form (LC3-II), mediated by the phosphorylation of PERK/eIF2 $\alpha$ , and the connection between autophagy and ER stress requires further investigation.

Inflammasome and autophagy are essential elements of the innate immune system, and their disruption has been implicated in the pathogenesis of type 2 diabetes. ER has previously been tightly linked to autophagy and inflammation, and has been considered as an intersection integrating multiple stress responses, and these processes are associated with the pathogenesis of diabetes mellitus and its complications. The results of the present study showed that the IRE1-mediated ER stress pathway activated the production of IL1 $\beta$ . Autophagy was also activated by the IRE1 pathway, which suggested that IRE1-mediated ER stress is essential for inflammatory cytokine secretion and the activation of autophagy. The results of the present study and previous findings, which link autophagy and inflammasome regulation, suggest that autophagy cooperates with inflammation and apoptosis, and the adaptive immune system, to orchestrate cellular homeostasis against danger signals, including ER stress. The data obtained in the present study, when combined with previous findings, indicate that there is a tight link between ER stress and inflammation autophagy, suggesting that a therapeutic strategy, which aims to target the common molecular processes altered in  $\beta$ -cells, may be effective.

In conclusion, the present study indicated that LPS induced an NLRP3-dependent proinflammatory response via excessive ER stress. Autophagy was shown to be important in ameliorating ER stress and NLRP3-dependent inflammatory cytokine secretion, further reducing INS-1 cell death. It was also shown that IRE1-pathway-mediated ER stress is a crucial link connecting autophagy with the NLRP3-dependent inflammatory response.

## Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81370929) and China Diabetes Young Scientific Talent Research Funding (2016).

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