ROS-mediated autophagy through the AMPK signaling pathway protects INS-1 cells from human islet amyloid polypeptide-induced cytotoxicity

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Abstract. Oligomerization of human islet amyloid polypeptide (hIAPP) is toxic and contributes to progressive reduction of β cell mass in patients with type 2 diabetes mellitus. Autophagy is a highly conserved homeostatic mechanism in eukaryotes. Previous studies have confirmed that hIAPP can promote autophagy in β cells, but the underlying molecular mechanism and cellular regulatory pathway of hIAPP-induced autophagy remains not fully elucidated. Accumulation of reactive oxygen species (ROS) causes hIAPP induced-β cell death. At present, little is known about the association between hIAPP-induced oxidative stress and autophagy in β cells. Therefore, the present study investigated the underlying molecular mechanism and regulatory pathway of hIAPP-induced autophagy. Transmission electron microscopy was used to observe the number of autophagosome in cells. Cell viability was determined by an MTT test. A 2',7'-dichlorofluorescin diacetate assay was used to measure the relative levels of reactive ROS. Western blotting was used to detect expression of adenosine monophosphate-activated protein kinase (AMPK) and autophagic markers p62 and microtubule associated protein 1 light chain 3. The results demonstrated that hIAPP induces autophagy through ROS-mediated AMPK signaling pathway in INS-1 cells. Upregulation of autophagy by AMPK activator 5-aminoimidazole-4-carboxamide1-β-D-ribofurano side decreased ROS and malondialdehyde generation, whereas inhibition of autophagy by 3-methyladenine and AMPK inhibitor compound C aggravated hIAPP-induced oxidative stress and toxicity in INS-1 cells. Taken together, the present study suggested that hIAPP induces autophagy via a ROS-mediated AMPK signaling pathway. Furthermore, autophagy serves as a cell-protective mechanism against hIAPP-induced toxicity and chemical promotion of autophagy through AMPK signaling pathway attenuates hIAPP induced cytotoxicity and oxidative stress in INS-1 cells.

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Introduction

Islet amyloid polypeptide (IAPP), also known as amylin, is a 37-amino-acid residue polypeptide (1), which is co-synthetized and secreted with insulin by β cells (2,3). Islet amyloid deposit, primarily derived from IAPP, is a pathological feature of 90% of patients with type 2 diabetes mellitus (T2DM) (4-7). Human-IAPP (hIAPP) has a propensity to form toxic oligomers spontaneously (1,5). Previous studies have demonstrated that aggregated hIAPP was toxic to islets and β cells *in vitro* or *in vivo* (8,9). Unlike hIAPP, rodent IAPP (rIAPP) that lacks β-sheet structure due to the 20-29 region proline substitutions, is nonamyloidogenic and nontoxic to β cells (10). The mechanisms of hIAPP-mediated toxicity are not yet completely elucidated. Therefore, further study of the underlying mechanisms of hIAPP-induced cytotoxicity in order to prevent loss of β cell mass is viewed as the clinical goal of treatment of T2DM.

As a result of imbalance between generation of reactive oxygen species (ROS) and antioxidant system (11), overproduction of ROS leads to oxidative stress. Previous studies have indicated that islet amyloid deposition induces oxidative stress and is associated with the decrease of β cell mass in patients with T2DM (4,12). *In vitro* studies also demonstrated that hIAPP promotes oxidative stress and that hIAPP-induced β cell death was alleviated by antioxidants (13,14). Redox state can regulate autophagy and ROS are generally accepted as inducers of autophagy (15). Autophagy is an evolutionarily conserved cellular mechanism for degradation of cytoplasmic components (16). Damaged organelles and abnormal proteins are sequestrated by autophagosomes (16) and subsequently transported to lysosomes for degradation and recycling (16). Under oxidative stress conditions, autophagy can degrade damaged mitochondria, which are important sources of intracellular ROS (17). Autophagy also removes oxidized proteins that are toxic to the cell (15). There is growing support for a hypothesis that autophagy is essential to maintain the function and mass of pancreatic β cells (18-20). Activation of autophagy by rapamycin relieved palmitate-induced damage to β cells (21). β cell specific disruption of autophagy associated gene 7 in mice

led to reduced insulin secretion, glucose intolerance and loss of β cell mass (20). Dysregulation of autophagy also serves a pathogenic role in amyloidosis-associated neurodegeneration, including Alzheimer's disease (22). However, in certain cases, the ROS scavenger catalase is also degraded by autophagy, therefore inhibition of autophagy decreases the accumulation of ROS and rescued cells from death (23,24). Therefore, the effect of autophagy on oxidative stress may be altered under different pathological conditions.

The above evidence indicated that autophagy may be involved in hIAPP-induced oxidative stress in β cells. The present study was designed to verify this hypothesis, and the results suggested that treatment with hIAPP promotes autophagic flux through ROS-mediated adenosine 5'-phosphate-activated protein kinase (AMPK) signaling pathway in INS-1 cells. Chemical activation of autophagy through AMPK signaling significantly attenuated hIAPP-induced INS-1 cell death and oxidative stress. Therefore, pharmacological modulation of autophagy through the AMPK signaling may offer an alternative therapeutic approach to prevent or slow β cell failure in T2DM.

Materials and methods

Cell line and regents. INS-1 cell line was purchased from Cell Center of Chinese Academy of Medical Sciences (Beijing, China). Compound C, AMPK activator 5-aminoimidazole-4-carboxamide1-β-D-ribofuranoside (AICAR), hIAPP, rIAPP, 3-methyladenine (3-MA), ammonium chloride (NH_4Cl) and MTT were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RPMI-1640 medium and fetal bovine serum (FBS) were from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). Microtubule-associated protein 1 light chain 3 mouse monoclonal antibody (LC3; cat. no. 2775; 1:1,000), phosphorylated (p)-AMPKα rabbit monoclonal antibody (Thr172; cat. no. 4188; 1:1,000), AMPKα1 rabbit monoclonal antibody (cat. no. 5831; 1:1,000), antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). P62 rabbit polycolonal antibody (cat. no. AF5384; 1:1,000) and β-actin mouse monoclonal antibody (cat. no. T0022; 1:3,000) were purchased from Affinity Biosciences (Cincinnati, OH, USA). Secondary monoclonal antibodies, including horseradish peroxidase (HRP)-labeled goat anti mouse immunoglobulin G (IgG; H+L; cat. no. E030120-01; 1:5,000) and HRP-labeled goat anti rabbit IgG (H+L; cat. no. E030110-01; 1:5,000) were purchased from EarthOx, LLC (Millbrae, CA, USA). ROS and malondialdehyde (MDA) content were measured with 2',7'-dichlorofluorescin diacetate (DCFHDA) assay kit and thiobarbituric acid (TBA) kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cell culture and intervention. INS-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mM mercaptoethanol, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 g/ml streptomycin and 100 U/ml penicillin at 37˚C in a humidified atmosphere containing 95% air and 5% CO_2. For treatment, hIAPP was dissolved in hexafluoroisopropanol (HFIP; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature (RT) for 8 h. Subsequently, HFIP was removed by evaporation under N_2 at RT for 1 h and stored at -80˚C. Prior to use, hIAPP was freshly dissolved in dimethylsulfoxide (DMSO) at 20 mg/ml and diluted to 10 μM by 20 mM PBS (pH 7.4). Cells were seeded in 96-well microplates at a density of $1x10^4$ cells/well for MTT assays. For other assays, cells were seeded in 6-well plates at a density of $2x10^5$/well. Cells were incubated with hIAPP (10 μM) or rIAPP (10 μM) for 24 h to examine the effects on INS-1 cells. For other treatments, cells were pretreated with 3-MA (0.5 nM), N-acetyl-L-cysteine (NAC; 20 mM), AICAR (2 mM), compound C (10 μM), NH_4Cl (5 mM) for 2 h and subsequently incubated with or without hIAPP (10 μM) for 24 h. The same volume of vehicle (DMSO <0.1%) was used as negative control.

Transmission electron microscopy (TEM). INS-1 cells were fixed at 4˚C in 2.5% glutaraldehyde in PBS overnight. Following fixation in 1% osmium tetroxide at 4˚C for 30 min and dehydration in 50, 70, 90 and 100% ethanol in turn at RT, each dehydration time was 15 min., Then cells were embedded in epoxy resin at 60˚C for 2 h. Subsequently, ultrathin section were obtained (~50-60 nm) and stained with 3% uranyl acetate at RT for 30 min. Images were captured by using HT7700 electron microscope (Hitachi, Ltd., Tokyo, Japan). Each group randomly selected 20 cells to count the number of intracellular autophagosomes.

MTT assay. Cell viability was measured by MTT assay. INS-1 cells were plated in 96-well microplates and cultured at 37˚C in a humidified atmosphere for 48 h and treated as described above. Following incubation, 20 μl/well MTT solution (5 mg/ml in PBS buffer) was added and incubated for 4 h. Following removal of the medium, 200 μl/well DMSO solution was added to dissolve the formazan crystals. Absorbance of soluble dye was measured at a wavelength of 570 nm with a microplate spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Measurement of ROS generation. Relative level of ROS in INS-1 cells was measured by DCFHDA assay. Cells were seeded in 6-well plates for corresponding treatment, as described above. Treated cells were washed with PBS and incubated with DCFHDA (10 mM) in phenol red free medium (GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C for 1 h. These cells were washed and resuspended with PBS at density of $1x10^5$ cells/ml. 2',7'-dichlorofluorescein (DCF) fluorescence was detected by an emission wavelength of 530 nm and excitation wavelength of 502 nm. Unlabeled cells at the same cell density in PBS were used as background control. The relative DCF fluorescence intensities of the treated cells were expressed as relative value to control. ROS in INS-1 cells were also directly observed and photographed by fluorescence microscopy in 6-well plates following incubation with DCFHDA for 1 h.

Determination of MDA. Following collection by centrifugation at RT for 5 min (250 x g), treated cells were washed with ice cold PBS and lysed with 1% trition X-100 (Beijing Leagene Biotech Co., Ltd., Beijing, China) on ice for 20 min. Subsequently TBA method was used to determine the levels of

MDA. This assay is based on the combination of MDA with TBA, which forms a red compound whose absorbance can by determined at a wavelength of 532 nm. The MDA content was expressed as nmol/mg protein.

Western blot analysis. Cells were harvested and washed with ice cold PBS, then homogenized in radio immunoprecipitation assay buffer (Solarbio Beijing Co., Ltd., Beijing, China). Lysates were mixed and incubated on ice for 10 min. Cell debris was precipitated at 4˚C for 10 min (16,099 x g). Protein concentrations were measured by bicinchoninic acid protein assay. Proteins were separated by 10% SDS-PAGE and electro-transferred to a polyvinylidene fluoride membrane. Nonspecific binding was blocked by incubation in 5% nonfat milk at RT for 2 h. Membranes were subsequently incubated with the primary antibodies at 4˚C overnight, washed 3 times with TBST (0.05% Tween-20) and finally incubated with a HRP-conjugated secondary antibody for 1 h. Protein bands were visualized using an chemiluminescence (ECL) kit (EMD Millipore, Billerica, MA, USA). Band intensities in the immunoblots were quantified by using image J program, version 1.48 (National Institutes of Health, Bethesda, MD, USA). P62 rabbit polycolonal antibody (cat. no. AF5384; dilution; 1:1,000) and β-actin mouse monoclonal antibody (cat. no. T0022; dilution, 1:3,000) were purchased from Affinity Biosciences.

Statistical analysis. Results were presented as the mean ± standard deviation. One-way analysis of variance with post hoc Tukey test was used for multiple comparisons by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

hIAPP induces autophagy in INS-1 cells. To verify the effect of hIAPP on autophagy in INS-1 cells, INS-1 cells were incubated in normal medium or medium containing hIAPP (10 μM) or rIAPP (10 μM) for 24 h. Consistent with a previous report (25), TEM observation demonstrated a significantly increased number of autophagosomes in hIAPP-treated INS-1 cells, compared with the control. However, cells treated with rIAPP exhibited no significant differences in the number of autophagosomes compared with the control group (Fig. 1A). Autophagy is a dynamic process and the number of autophagosomes at a certain time point depends on a balance between autophagosome generation and degradation (26). Increase of autophagosome number may be either result from a decrease in lysosome degradation or enhanced autophagosome formation (26).

Subsequently, western blot analysis was used to evaluate autophagic markers LC3 and p62. LC3 is processed into a soluble form LC3-I and subsequently LC3-I is converted to LC3-II, which is specifically located in autophagic membrane (27). Therefore, the conversion of LC3-I to LC3-II may reflect level of autophagy (28). P62 is incorporated into autophagosomes by binding to LC3 and is degraded by autophagy (28). Therefore, the amount of p62 exhibits a negative association with autophagic activity. INS-1 cells were treated with rIAPP (10 μM) or hIAPP (10 μM), with or without NH_4Cl for 24 h and the corresponding vehicle (DMSO <0.1%) was used as control. Treatment with hIAPP resulted in a significant increase of LC3-II expression. NH_4Cl is a lysosomal inhibitor, which could inhibit the degradation of autophagosomes (Fig. 1B). Co-incubation of NH_4Cl and hIAPP could further increase LC3-II expression (Fig. 1B). By contrast, the level of p62 was decreased in INS-1 cells treated with hIAPP. Combined treatment with hIAPP and NH_4Cl resulted in accumulation of p62 (Fig. 1B). All above results support the hypothesis that hIAPP induces autophagy in INS-1 cells.

Involvement of ROS in hIAPP-stimulated autophagy. Previous studies have demonstrated that ROS are upstream modulators of autophagy (15,29). To determine the effect of hIAPP on ROS generation by INS-1 cells, INS-1 cells were treated with NAC (20 mM), hIAPP (10 μM), or NAC (20 mM) and hIAPP (10 μM) for 24 h, and corresponding vehicle (DMSO <0.1%) was used as control. Intracellular ROS level was determined by DCFHDA assay. hIAPP enhanced intracellular ROS generation in INS-1 cells, compared with the control (Fig. 2A). Antioxidant NAC alone did not alter ROS levels compared with the control groups. Co-treatment with NAC significantly inhibited ROS accumulation in hIAPP-treated INS-1 cells, compared with the hIAPP group ($P<0.01$; Fig. 2A). To determine the effect of hIAPP-induced oxidative stress on autophagy in INS-1 cells, western blots were used and probed for LC3-II expression. NAC alone did not alter LC3-II expression, but it significantly suppressed hIAPP-induced LC3-II expression (Fig. 2B). These results confirmed that oxidative stress serves a role in hIAPP-induced autophagy.

Effects of AMPK signaling pathway on hIAPP-induced autophagy in INS-1 cells. Although connections between oxidative stress and autophagy have been extensively studied (30,31), the signaling pathway that links these processes in β cells has not been examined in detail. Previous studies have suggested that overproduction of ROS induces AMPK activation (32), activation of unc-51 like autophagy activating kinase 1 and inhibition of mTOR complex 1 by AMPK, leading to initiation of autophagy (33,34). Western blots demonstrated that treatment with NAC or rIAPP alone did not alter the level of AMPK phosphorylation (Fig. 3A). However, treatment with hIAPP led to enhancement of AMPK phosphorylaiton at Thr172. These results indicated that AMPK signaling pathway was activated by hIAPP in INS-1 cells. However, this effect was partly inhibited by co-culture with NAC (Fig. 3A), suggesting that ROS may be involved in hIAPP-induced AMPK activation. Similar to hIAPP, AMPK activator AICAR also enhanced LC3-I to LC3-II conversion in INS-1 cells (Fig. 3B). However, both hIAPP- and AICAR-induced LC3-II turnover was inhibited by AMPK inhibitor compound C. These results suggested that AMPK serves a role in regulation of autophagy in hIAPP-treated INS-1 cells.

Activation of autophagy through AMPK signaling attenuates hIAPP-induced cytotoxicity and oxidative injury in INS-1 cells. Inhibitor of autophagy 3-MA was used to study the effects of autophagy on hIAPP-induced cytotoxicity and oxidative damage. Treatment of INS-1 cells with 10 μM hIAPP for

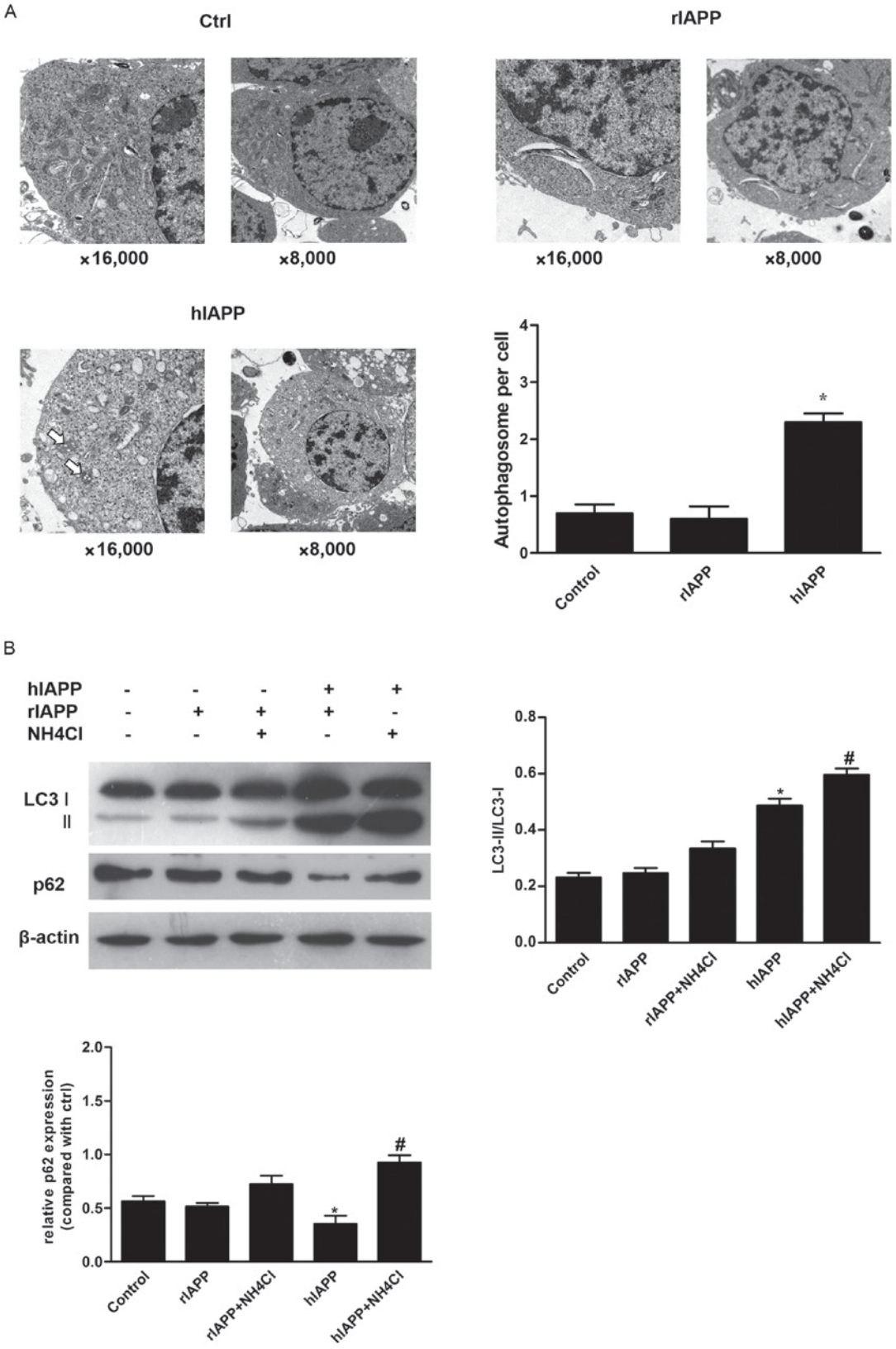

Figure 1. hIAPP induces autophagy in INS-1 cells. (A) Representative electron microphotographs and statistical data (the average number of autophagosome in each cell) are presented. White arrows indicate autophagosomes in INS-1 cells. *P<0.05 vs. the control group. (B) Western blots were conducted to determine LC3-II and p62 expression, β-actin expression was detected as loading control. Quantified data of blots are presented as the mean ± standard deviation of three separate experiments. *P<0.05 vs. the control group. #P<0.05 vs. the hIAPP group. hIAPP, human islet amyloid polypeptide; rIAPP, rodent islet amyloid polypeptide; LC3, microtubule associated protein 1 light chain 3; ctrl, control group.

24 h resulted a significant decrease in cell viability compared with the control (Fig. 4A). Light microscopy observation also demonstrated that treatment with hIAPP resulted in morphological alterations compared with the control group, including oval

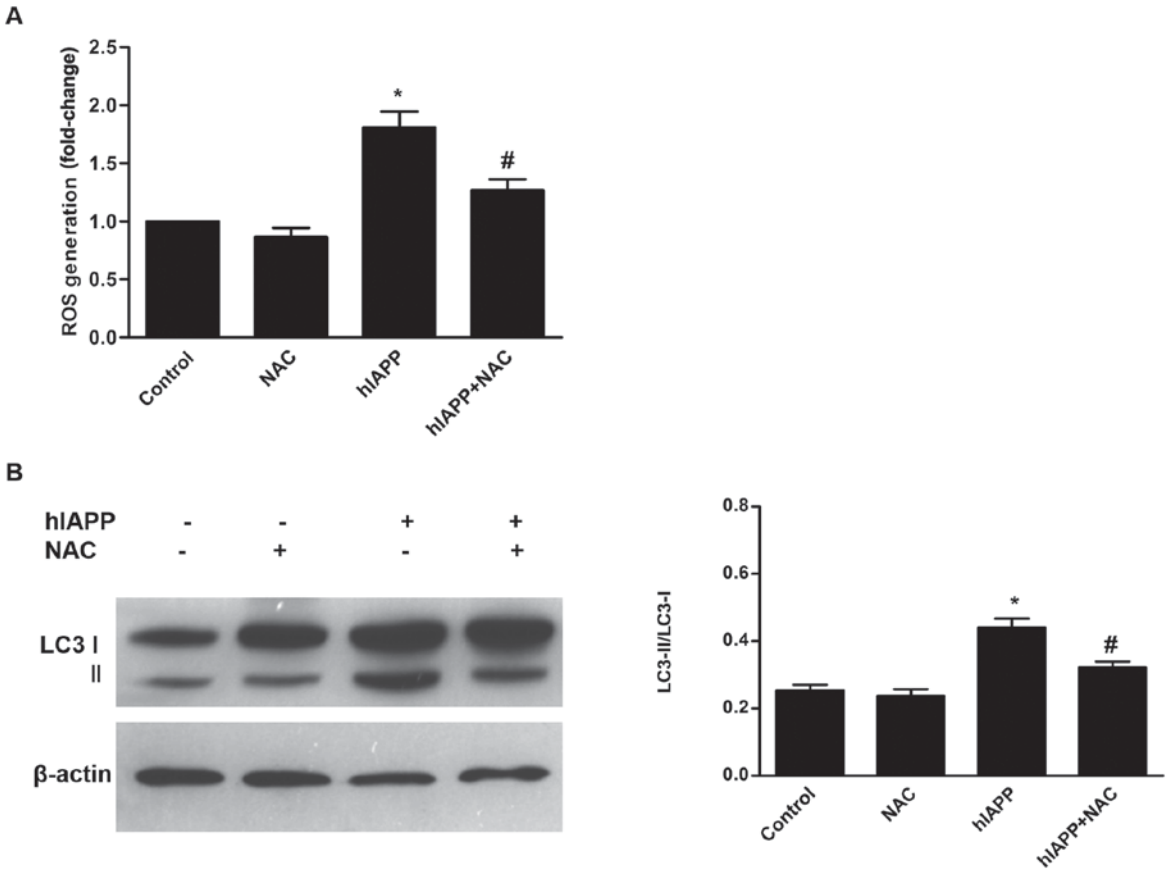

Figure 2. Involvement of ROS in hIAPP-induced autophagy. (A) ROS generation was determined by measuring fluorescence intensity of DCF. Intracellular levels of ROS were expressed as relative ratio of control (valued as 1). *P<0.05 vs. the control group. #P<0.05 vs. the hIAPP group. (B) Western blots were conducted to determine LC3-II expression, β-actin expression was detected as loading control. Quantified data of blots are presented as the mean ± standard deviation of three independent repeats. *P<0.05 vs. the control group. #P<0.05 vs. the the hIAPP group. ROS, reactive oxygen species; LC3, microtubule associated protein 1 light chain3; NAC, N-acetyl-L-cysteine; hIAPP, human islet amyloid polypeptide.

shape, shrinkage, blebbing and detachment from the culture dish. 3-MA alone exhibited minimal effect on cell survival. However, 3-MA significantly enhanced hIAPP-induced cell viability loss and the extent of above morphological alterations of INS-1 cells. The levels of ROS and MDA were measured as markers of oxidative stress. Consistent with previous studies (13,35), treatment of INS-1 cells with hIAPP resulted in elevated levels of ROS and MDA compared with the control group (Fig. 4B). Co-treatment with 3-MA further enhanced intracellular ROS and MDA generation in hIAPP-treated cells.

Subsequently, the present study investigated the effects of chemical modulation of autophagy through AMPK signaling on hIAPP-induced cytotoxicity and oxidative damage. INS-1 cells were treated with hIAPP in the presence or absence of compound C or AICAR. Compound C or AICAR exhibited no effect on cell viability or oxidative stress in INS-1 cells (Fig. 4). Pretreatment with compound C caused greater extent of cell death and elevated levels of ROS and MDA in hIAPP-treated INS-1 cells (Fig. 4). By contrast, pretreatment of INS-1 cells with AICAR partially prevented hIAPP-induced cytotoxicity and oxidative stress (Fig. 4). However, these protective effects of AICAR were abolished by the presence of 3MA (Fig. 4B). The effects of different treatments on ROS generation in INS-1 cells were also visualized by fluorescent microscopy. The above result findings were further confirmed by the DCF fluorescence of each group (Fig. 4C). Together these results suggested that chemical activation of AMPK in INS-1 cells promotes survival against hIAPP-indcuced oxidative damage and this effect likely occurs through induction of autophagy.

Discussion

Islet amyloid deposition is a characteristic histopathological feature of patients with T2DM (18). The main component of islet amyloid is hIAPP. Evidence suggests that hIAPP contributes to β cell dysfunction and death in T2DM (4-7). Autophagy is a self-digestion system used to maintain cellular homeostasis through degradation and recycling of cellular components (16). Dysregulated autophagy has been suggested to be involved in the development of β cell failure associated with T2DM (20). It has been reported that autophagy serves a role in hIAPP-induced toxicity of β cells (25), but the underlying mechanism and cellular regulatory pathway of hIAPP-induced autophagy remains to be completely elucidated. The present study investigated how hIAPP induces autophagy and the effects of chemical modulation of autophagy through the AMPK pathway on the oxidative injury induced by hIAPP in β cells. In agreement with previous studies (23,25), the present results demonstrated that hIAPP promotes autophagy in INS-1 cells. Electron micrographs indicated that INS-1 cells following treatment with hIAPP exhibited an increased number of autophagosomes compared with control cells. Treatment with

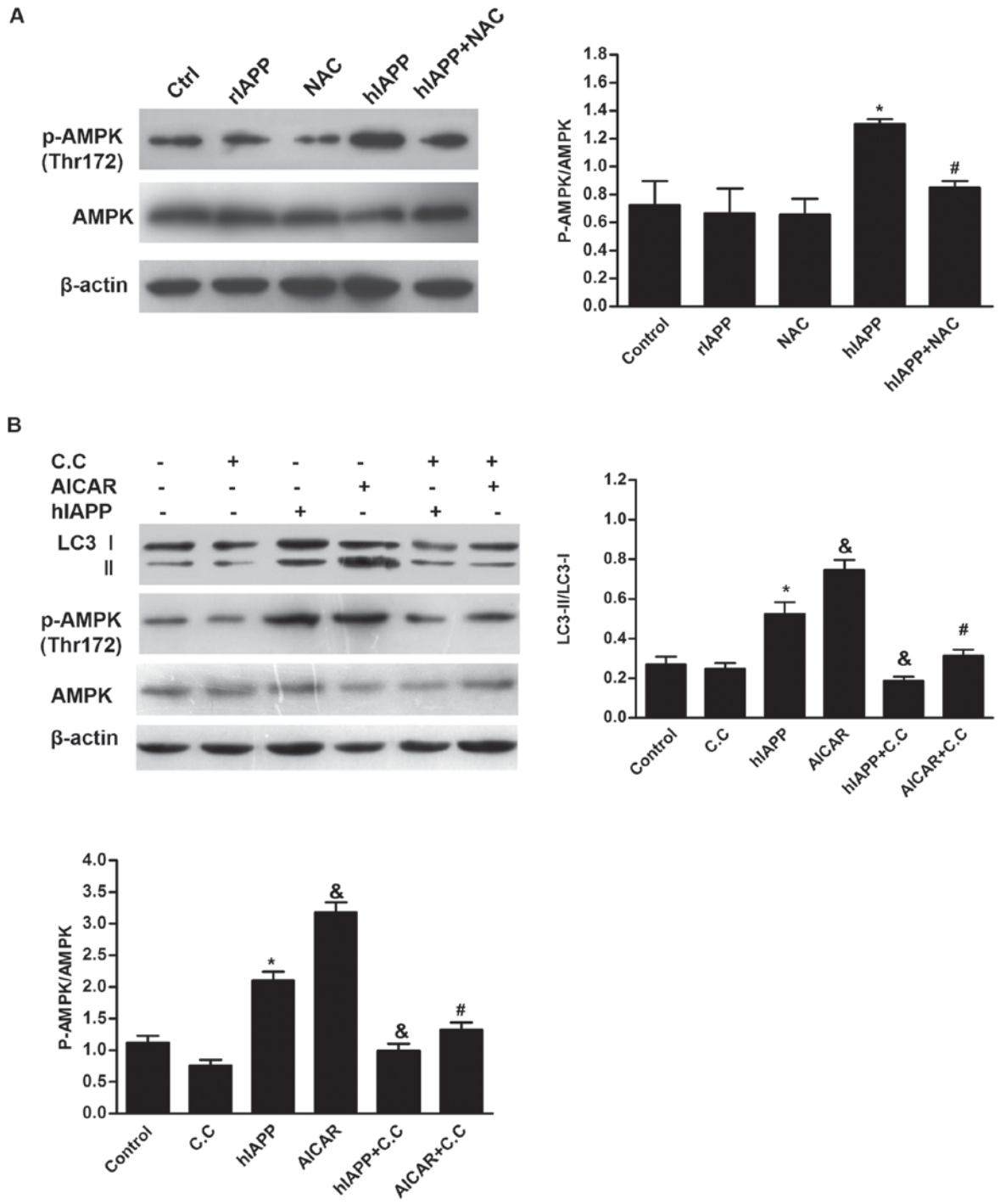

Figure 3. hIAPP promotes autophagy through AMPK signaling pathway. (A) Western blots were conducted to determine expression of p-AMPK and AMPK. *P<0.05 vs. the control group. #P<0.05 vs. the hIAPP group. (B) Western blots were conducted to determine expression of p-AMPK, AMPK and LC3-II following different treatments. β-actin expression was detected as loading control. Quantified data of blots is expressed presented as the mean ± standard deviation of three independent repeats. *P<0.05 vs. the control group. &P<0.05 vs. the the hIAPP group. #P<0.05 vs. the AICAR group. AMPK, adenosine 5'-phosphate-activated protein kinase; AICAR, AMPK activator 5-aminoimidazole-4-carboxamide1-β-D-ribofuranoside; C.C, compound C; p, phosphorylated; hIAPP, human islet amyloid polypeptide; rIAPP, rodent islet amyloid polypeptide; NAC, N-acetyl-L-cysteine.

hIAPP also enhanced the conversion of LC3-I to LC3-II and was accompanied by decreased expression of p62 in INS-1 cells.

Subsequently, the molecular mechanism of hIAPP-induced autophagy was investigated. It has been previously indicated that enhanced oxidative stress is associated with the extent of islet amyloid deposition and β cell mass in T2DM (4). ROS may act as initiators for hIAPP-induced cytotoxicity in β cells (14,35). It is also commonly accepted that ROS induce autophagy (36). To confirm the role of oxidative stress in the induction of hIAPP-induced autophagy, INS-1 cells were treated with hIAPP in the presence or absence of NAC for 24 h. Consistent with previous studies (13,37), the present study demonstrated that hIAPP significantly enhanced ROS generation and this effect was attenuated by treatment with antioxidant NAC. Treatment with NAC alone induced no effect on LC3-II expression, but it partly inhibited LC3-II expression

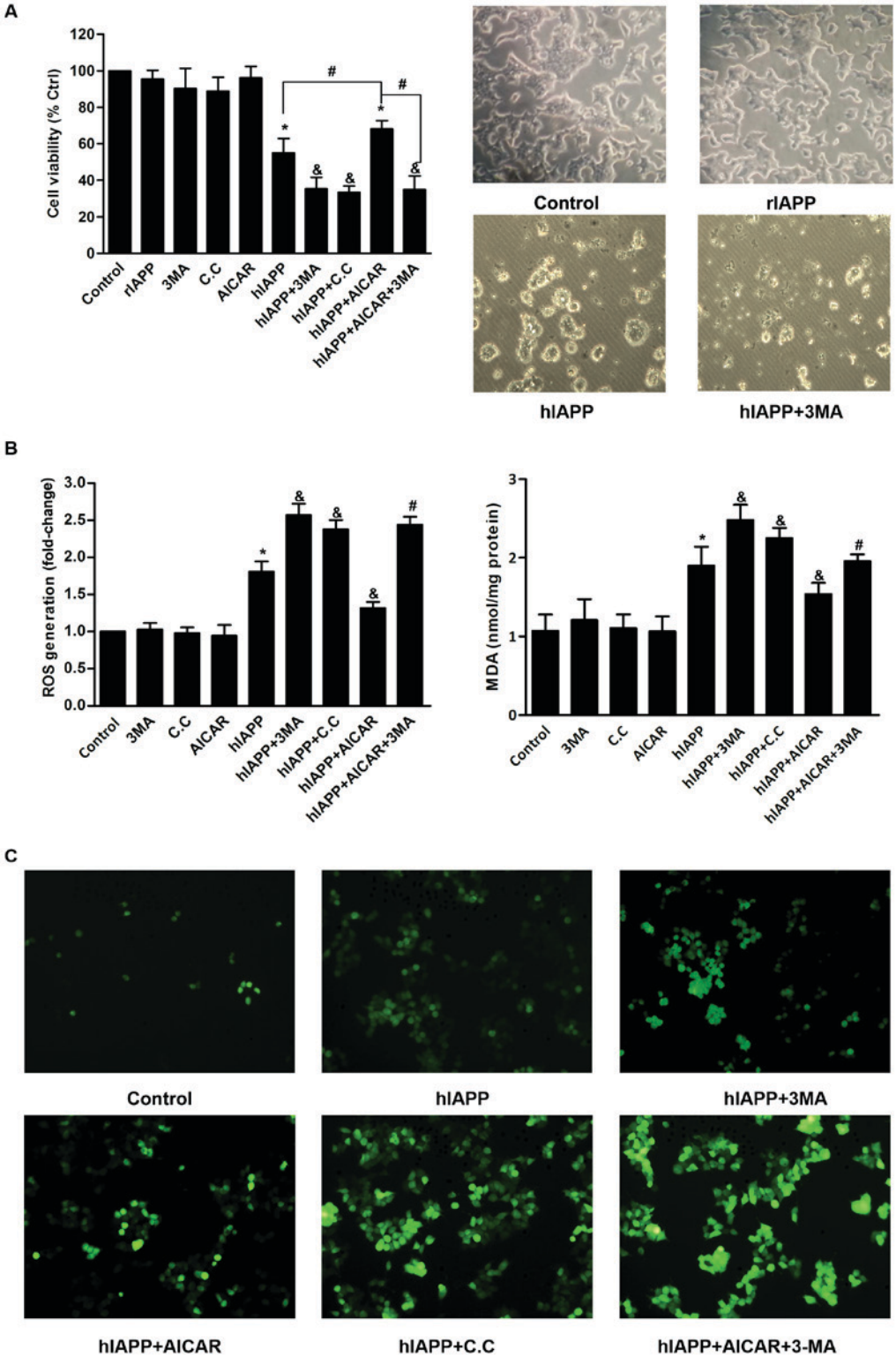

Figure 4. Activation of autophagy through AMPK signaling attenuates hIAPP-induced cytotoxicity and oxidative injury. (A) Cell viability was determined by MTT assay. All data are expressed as the mean ± standard deviation from five independent experiments. *P<0.05 vs. the control group. &P<0.05 vs. the hIAPP group. #P<0.05. The morphology of INS-1 cells was observed and images were captured under a microscope (magnification, x160). Representative morphologic images were presented. (B) Intracellular ROS and MDA were determined according to the manufacturer's protocol using a spectrophotometer. Intracellular levels of ROS were expressed as relative ratio of control (valued as 1). All data are expressed as the mean ± standard deviation from three independent experiments. *P<0.05 vs. the control group. &P<0.05 vs. the hIAPP group. #P<0.05 vs. the hIAPP+AICAR group. (C) Intracellular ROS levels were also observed by fluorescence microscopy (magnification, x100). The green fluorescence of DCF represents the levels of intracellular ROS. AMPK, adenosine 5'-phosphate-activated protein kinase; AICAR, AMPK activator 5-aminoimidazole-4-carboxamide1-β-D-ribofuranoside; MDA, malondialdehyde; 3-MA, 3-methyladenine; ROS, reactive oxygen species; hIAPP, human islet amyloid polypeptide; rIAPP, rodent islet amyloid polypeptide; ctrl, control; C.C, compound C.

in hIAPP-treated INS-1 cells. It can therefore be concluded that hIAPP induces autophagy through generation of ROS.

ROS can activate a series of cell signaling pathways involved in various cellular processes. The present understanding of

molecular mechanisms, signaling pathways and the redox reactions regulated by autophagy is incomplete. AMPK is known as a sensor of alterations in energy metabolism and redox state (38). Recent studies have demonstrated that AMPK is a regulator of autophagy and can activate tuberous sclersis complex 1/2, leading to inhibition of mTOR pathway and initiation of autophagy (32,39,40). However, there is also evidence indicating that AMPK may negatively regulate autophagy in β cells (41), suggesting that regulation of autophagy by AMPK depends on the cell type and environment. In the present study, treatment with hIAPP significantly promoted AMPK phosphorylation. Similar results were not obtained following treatment with rIAPP, indicating that the induction of AMPK phosphorylation is not the effect of IAPP monomers combining with cellular receptors. Furthermore, it was observed that hIAPP-induced AMPK phosphorylation was partly inhibited by co-treatment with NAC, suggesting that AMPK activation may be induced by ROS. Inhibition of AMPK by compound C reduced the amount of LC3-II in hIAPP-treated INS-1 cells. AMPK activator AICAR could further promote LC3-II expression. Based on these results, we hypothesized that AMPK is major signaling pathway involved in activation of INS-1 cell autophagy.

Autophagy can induce both positive and negative effects and although it is generally regarded as a protector against stressors, excessive autophagy can also lead to non-apoptotic cell death. Oxidative stress is a mediator of hIAPP-induced cytotoxicity in β cells (4,13,14). In the present study, treatment of INS-1cells with hIAPP also enhanced intracellular ROS and MDA generation. Accumulation of ROS can cause DNA damage, protein denaturation and lipid peroxidation (42). MDA is an end products of lipid peroxidation and it can alter the structure and function of the cell membrane and inhibit cellular metabolism, leading to cytotoxicity (42). Therefore, MDA is commonly used as a marker for oxidative stress (42). Because of relatively low levels of antioxidants, including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), β cells are susceptible to oxidative damage (43). ROS have been proposed as initiators of hIAPP-induced toxicity. Damaged organelles, including mitochondrion and endoplasmic reticulum are the main sources of ROS during cellular stress (17). Autophagy can selectively remove these obsolete organelles in order to limit ROS amplification (15). Oxidized damaged proteins, which are toxic to β cells are also degraded by autophagy. In certain cases, the ROS scavenger catalase also undergoes protein degradation via autophagy and inhibition of autophagy reduces accumulation of ROS and rescues cells from death (23,24). Therefore the effect of autophagy on oxidative stress may be altered under different pathological conditions. In the present study, it was demonstrated that inhibition of autophagy or AMPK exacerbates oxidative stress and renders INS-1 cells more susceptible to hIAPP-induced toxicity. By contrast, stimulating autophagy by AMPK activator AICAR alleviated hIAPP-induced cell death and oxidative stress in INS-1 cells. The protective effect of AICAR was abolished by co-treatment with 3-MA. Therefore, chemical activation of autophagy through AMPK may limit hIAPP-induced oxidative injury in INS-1 cells.

In conclusion, the present study demonstrated that hIAPP promotes autophagy via ROS mediated AMPK signaling pathway in INS-1 cells. Autophagy may act as an antioxidative mechanism to antagonize hIAPP-induced cytotoxicity in β cells. Chemical activation of autophagy through AMPK signaling attenuates hIAPP-induced oxidative injury in INS-1 cells. AMPK is the target of numerous agents including certain hypoglycemic drugs (44,45). Although the present study limited to *in vitro* experiments, similar mechanisms may act in hIAPP-mediated cellular dysfunction of human β cells. These results also suggest that pharmacological modulation of autophagy through AMPK may be a therapeutic target to conserve β cell mass in the initiation and progression of T2DM.

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Availability of data and materials

The datasets used and analyzed during the current study are available from corresponding author on reasonable request.

Authors' contributions

GX and TZ conceived and designed the study. GX, XL, YJ, JZ and JX performed the experiments. GX and TZ wrote the paper. TZ reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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