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Exendin-4 impairs the autophagic flux to induce apoptosis in pancreatic acinar AR42J cells by down-regulating LAMP-2

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ABSTRACT

This study aimed to explore the mechanism of impaired autophagy flux induced by exendin-4 and its role on cell apoptosis in pancreatic AR42J cells. The AR42J cells were treated with various concentration of exendin-4 for several time points to assess its cytotoxicity by MTT assay. Then the AR42J cells were treated by 10pM exendin-4 for 72 h, the cell death was analyzed by flow cytometry and caspase-3 level was examined by Western blot with or without the pretreatment of z-VAD-fmk to testify whether exendin-4 induces the cell apoptosis. The protein levels of LC3B, p62 and LAMP-2 were assessed by Western blot, the mRNA level of LAMP-2 was quantified by quantitative PCR in the absence or presence of LAMP-2 over-expression plasmid and the expression and activity of CatB and CatL were tested by ELISA or activity assay methods in AR42J cells treated by exendin-4. The normal rats and the diabetes-model rats by high-fat and high-sugar diet for two month then with streptozotocin intraperitoneally were subcutaneously injected with exendin-4 for 10 weeks to test the expression of LAMP-2 mRNA and protein in the pancreas. Cells pretreated with Bafilomycin A1 were detected for LC3B and p62 expressions by Western blot. Cells pretreated by 3-MA were used to assess whether 3-MA can protect from exendin-4 cytotoxicity. We found that exendin-4 can decrease the AR42J cell viability as well as increase the cell death and cleaved caspase-3 level, which all can be inhibited by z-VAD-fmk. Exendin-4 can downregulate the expression of LAMP-2 and then impair the autophagy flux to induce the accumulation of LC3B-II and p62, but cannot change the expression and activity of CatB and CatL. Bafilomycin A1 almostly have no impact on the change of LC3B and p62 protein levels induced by exendin-4. Both 3-MA and overexpressed LAMP-2 can reduce the cytotoxicity of exendin-4. Therefore, we considered the down-regulation of LAMP-2 which can impair the autophagy flux by inhibiting the fusion of autophagosomes with lysosomes to induce the AR42J cell apoptosis as the potential mechanism of chronic pancreatitis induced by exendin-4.

1. Introduction

Exendin-4 (Ex-4, its synthetic version named exenatide) is a human glucagon-like peptide 1 (GLP-1) analogue approved firstly to treat type-2 diabetes by FDA in 2005. After its clinical treatment, some doctors found that Ex-4 may be a reason for the admission of acute pancreatitis [1–4]. Although there has always be a controversy on whether Ex-4 can induce acute pancreatitis, the damage to pancreas induced by Ex-4 has been observed in some animal experiments. Koehler et al. [5] found that chronic Ex-4 treatment increased the pancreatic mass in high-fat–fed mice and selectively modulated the expression of some pancreatitis associated genes.

Abbreviations: Ex-4, Exendin-4; GLP-1, glucagon-like peptide 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; LC3B, light chain 3B; LAMP-2, lysosome associated membrance protein; CatB/L, cathepsin B/L; 3-MA, 3-methyladenine; Baf-A1, Bafilomycin A1; PI-3K, phosphatidylinositol-3 kinase; DM, diabetes model rat.

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Nachnani et al. [6] found that extended treatment of Ex-4 induced the pancreatic acinar inflammation and cell pyknosis in rats. Gier et al. [7] found that extended Ex-4–treated rats demonstrated scant intact acini with extensive inflammation and fibrosis. Our previous studies also showed that extended Ex-4 treatment in rats caused chronic pancreatitis including pancreatic acinar pyknosis and vacuolization [8,9]. To explore the mechanism of pancreatic acinar pyknosis and vacuolization induced by Ex-4, we further found that Ex-4 can induce pancreatic acinar cell apoptosis which was consistent with pancreatic acinar pyknosis and impair the autophagic flux which resulted in vacuolization [10]. However, whether there is any relation between vacuolization and the cell apoptosis is unclear.

Macroautophagy (herein referred to as autophagy) is the fundamental cellular mechanism of lysosome-driven degradation which recycles cellular organelles and long-lived proteins. Under the situations such as starvation, stress and some pathophysiological factors, autophagy will sequester materials destined for degradation into autophagosome, which then fuses with a lysosome to form autolysosome where sequestered materials are degradated to suppy the cell's energy and biogenesis needs. The process of autophagy is termed as autophagy flux, which will be impaired because of blockage of autophagosome-lysosome fusion or lysosome dysfunction to make autophagosome accumulate and vacuolization in cell cytoplasm. We previously found that Ex-4 can damage the pancreatic acinar cells by impairing the autophagic flux to result in autophagosome accumulation [10]. Impaired autophagy flux has been shown to induce cell apoptosis by activating certain cell signaling pathway [11–13], so we hypothesized that Ex-4 can trigger pancreatic acinar cell apoptosis to damage the pancreas by impairing autophagic flux. This study is to explore how Ex-4 impairs autophagic flux and whether impaired autophagic flux can trigger pancreatic acinar cell apoptosis.

2. Materials and methods

2.1. Animals experiment

The animal experimental protocols were approved by the University Animal Care and Use Committee of the Association for Assessment and Accreditation of Laboratory Animal Care. Thirty of fifty male SD rats (Hunan Slacjingda LabAnimal Inc, Changsha, China) weighing 180–210 g were randomly selected as the diabetes-model rats which were fed with a high-sugar and high-fat diet for 2 months and subsequently were injected intraperitoneally with a dose of 35 mg/kg streptozotocin (Sigma, St. Louis, MO, USA). Three days later, the fasting blood glucose level (FBG) was tested to select the successful model, of which the FBG should be higher than 16.7 mmol/L. The FBG was measured weekly to exclude any rats of which the FBG was less than 16.7 mmol/L for 3 times continually. Meanwhile, the other twenty normal SD rats were fed normally for 2 months. The diabetes-model rats and the normal rats were divided into two subgroups respectively, one group was treated with exenatide (GL Biochem Shanghai Ltd, Shanghai, China) by injecting subcutaneously at a dosage of 5 μg/kg at 8:00 A.M. and 6:00 P.M. of each day and the other group was treated with normal saline as control. Ten weeks later, the pancreases were harvested to detect the expression of LAMP-2.

2.2. Cell culture

As previously reported [10], pancreatic acinar cells line AR42J cells (American Type Culture Collection, Manassas, Va) were cultured in F-12K medium with 2 mM L-glutamine, 250 μg/mL amphotericin, double antibiotics (1% of 100 U/mL penicillin and 1% of 100 U/mL streptomycin) and 20% fetal bovine serum, and incubated in a 5% CO_2 and 37 °C atmosphere at a density of approximately 2×10^5 cells/mL in 6-well plates.

2.3. Plasmids and transfection

The LAMP-2 overexpression plasmid pRP-rLamp2 (pRP(exp)-EGFP/Puro-CAG > rLamp2, VB170911- 1011xeb) and the empty vector (pRP(exp)-Puro-CAG > EGFP, VB170911-1017bpf) were obtained from VectorBuilder. The transfection was performed using Lipofectamine 2000 (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions.

2.4. Cell viability assay by MTT

AR42J cells were seeded into 96-well plates and allowed to attach for 24 h. The cells were then treated with increasing concentrations of Ex-4 (0, 1, 5 and 10 pmol/L) and incubated for 24, 48, 72, 96 and 120 h, respectively. After the incubation, 20 μl MTT (Sigma, St. Louis, MO, USA) was added and cells were incubated for another 4 h. After removing the supernatants, crystals were lyzed with 100 μl DMSO and the absorbance was meatured at a wavelength of 570 nm with background subtraction at 690 nm in a GENios spectrofluorimeter (Tecan, Salzburg, Austria). Five individual wells were prepared for each treatment concentration. The percentage cell viability was calculated as the ratio of mean absorbance (from three middle absorbances of five wells) of treated versus untreated cells.

2.5. Cell apoptosis analysis by flow cytometry

Cell apoptosis was analyzed by flow cytometry with FITC/AnnexinV Apoptosis Detection Kit I or PE/AnnexinV Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ, USA). Green fluorescence in the transfected AR42J cell lines overlaps with FITC, so the cell apoptosis of transfected AR42J cell lines was detected by PE/AnnexinV Apoptosis Detection Kit I. After cultured with 10 pM Ex-4 for 72 h, the floating cells and adherent cells trypsinized were collected together, then treated according to the manufacturer's instructions and analyzed by flow cytometry (BD FACSCalibur™, Becton Dickinson, San Jose, CA, USA). Quandrant statistics of FL2-H/FL1-H dot plot was carried out using Flowjo software (FlowJo, LLC, Ashland, OR, USA). Cell apoptosis was determined as the percentage of cells stained positive of AnnexinV or both.

2.6. Protein expression detected by Western blot

The pancreatic tissues or treated AR42J cells were lysed and centrifuged, then the proteins were extracted from the supernatant, separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, and probed by antibodies. The membranes were incubated with microtubule-associated protein light chain 3B (LC3B) specific antibody (18725-1-AP, Proteintech), p62/SQSTM1 antibody (18420-1-AP, Proteintech), LAMP-2 antibody (10397-1-AP, Proteintech), Caspase-3 antibody (ab4051, Abcam, UK) and β-actin antibody (60008-1-Ig, Proteintech, as loading control) overnight at 4 °C. Then the membranes were incubated with the corresponding secondary antibodies (goat-anti-mouse IgG, SA00001-1, Proteintech; goat-anti-rabbit IgG, SA00001-2, Proteintech) for 2 h. The blots were detected by enhanced chemiluminescence (ECL, BIO-RAD, Hercules, CA, USA) on X-ray film. Densitometric analyses were conducted with Quantity One software (BIO-RAD, Hercules, CA, USA) and data were expressed in arbitrary units.

2.7. LAMP-2 mRNA level by quantitative PCR

Total RNA was extracted from fresh rat pancreatic tissue using RNeasy® mini kit (Qiagen Inc., Valencia, CA, USA) or treated AR42J cell lines. RNA concentration was quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Then 2.0 mg of each RNA sample was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). All procedures were performed according to the manufacturer's instructions. Quantitative real-time PCR was performed on ABI Prism 7900 HT Sequence Detection System using TaqMan Universal PCR Master Mix, and GAPDH was used as internal control (all from Applied Biosystems). Primers are as follow: LAMP-2: 5′-CATTGGGTGTCATCTTTAAGTGC-3′, 5′-GAATGATGGGTGCTACAGTGGT-3'; GAPDH: 5′-AGTGCCAGCCTCGTCTCATA-3′, 5′- ATCCGTTCACACCGACCTTC-3'. The mRNA expression level was shown as the relative fold of change, normalized by that of GAPDH.

2.8. CatB and CatL ELISA assay

Cells were lysed and centrifuged, and the expression levels of cathepsin (Cat)B and CatL including the precursors, the active forms of cathepsins as well as cathepsin complexes with endogenous inhibitors in the supernatant, were tested by CatB and CatL sandwich ELISA kits (Prof. Janko Kos, Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia). Protein samples were diluted to 20 mg protein/ml (for CatB) or 80 mg protein/ml (for CatL). Then horseradish peroxidase-conjugated secondary antibody was added to measure the absorbance of a chromogenic product at 450 nm. Results were expressed in nM of specific protein based on a calibration curve and presented as mean of three independent experiments.

2.9. CatB and CatL activity assay

Cells were lysed and centrifuged, then the supernatant samples were diluted by distilled water and activated at 37 °C for 30 min in activation buffer [buffer A: 0.4 M phosphate buffer (pH6.0) and 2.5 mM fresh dithioerythritol (DTE) for CatB; buffer B: 0.34 M acetate buffer (pH 4.2) and 2.0 mM fresh DTE for CatL; all reagents were obtained from Sigma-Aldrich]. Next, the activated samples reacted at 37 °C in reaction buffer [buffer A for CatB; buffer C: 0.34 M acetate buffer (pH 5.5) and 2.5 mM fresh DTE, for CatL] in the presence or absence of certain inhibitor (60 μM Ca-074, Peptide Institute, Osaka, Japan, for CatB; 2 μM Clik148, provided by N. Katunuma, Tokushima Bunri University, Tokyo, Japan, for CatL), and in the presence of certain fluorogenic substrate (100 μM Z-Arg-Arg-AMC for CatB, 100 μM Z-Phe-Arg-AMC for CatL; both were purchased from Bachem, Bubendorf, Switzerland). After 90 min, the reaction was stopped by using iodoacetic acid (1 mM), and the released 7-AMC was measured (360 ± 35 nm excitation and 465 ± 35 nm emission) on a spectrofluorimeter (Tecan GENios, Groedig, Austria) and quantified based on an AMC calibration curve. Certain cathepsin activity was reported as ($\mu mol_{AMC\ released}/mg_{total\ protein} \times min$) and mean of three independent experiments was presented.

2.10. Statistical analysis

All data were expressed as the mean ± standard deviation (SD) and analyzed by unpaired Student *t*-test or two-way ANOVA which only was used to analyze the result of MTT by SPSS 18.0 statistics software (SPSS China, Shanghai, China). The value of $P < .05$ was considered statistically significant for all tests. All experiments were done in triplicate.

3. Results

3.1. Ex-4 induced the AR42J cell apoptosis

As shown in Fig. 1a, Ex-4 inhibited the AR42J cell viability in a dose-time dependent manner. To examined whether the inhibitory effect of Ex-4 was associated with the induction of apoptosis, we testified the induction of apoptosis by flow cytometry and determined the expression of caspase-3 after treatment with 10 pM Ex-4 for 72 h. Ex-4 notably induced the cell apoptosis, showing significant increase in the percentage of apoptosis cells in flow cytometry (Fig. 1c) and up-regulation of cleaved caspase-3 (Fig. 1d). Further, pretreatment with z-VAD-fmk (apoptosis inhibitor, 100 μM, Selleck Chemicals, USA) for 30 min can effectively inhibit the viability reduction (Fig. 1b) and cell apoptosis (Fig. 1c and d).

3.2. Ex-4 impaired the autophagic flux by downregulating LAMP-2

After exposing AR42J cells in 10 pM Ex-4 for 72 h, the protein expressions of LC3B and p62 were seen to significantly increase, and the protein and mRNA expressions of LAMP-2 were significantly downregulated, as shown in Fig. 2a. Similarly, LAMP-2 protein and mRNA expressions were also significantly downregulated in the diabetes-model rats as well as normal rats with long-term treatment of exenatide (Fig. 2b). However, treatment of Ex-4 cannot affect the expression and activity of CatB and CatL in the AR42J cells (Fig. 2c). Furthermore, pretreatment with Bafilomycin A1 (Baf-A1, 50 nM, Sigma-Aldrich, USA), which is a vacuolar type H^+-ATPase inhibitor to prevent the acidification of lysosome and consequently to abolish the fusion of autophagosome with lysosome thus inducing the LC3B-II and p62, accumulation, could not augment the accumulation of LC3B and p62 induced by Ex-4 (Fig. 2d).

3.3. LAMP-2 overexpression reversed the impaired autophagic flux and apoptosis induced by Ex-4

To further verify that LAMP-2 down-regulation was the main reason of impaired autophagic flux which resulted in cell apoptosis induced by Ex-4, we overexpressed LAMP-2 by transfecting pRP-rLamp-2 plasmid into the AR42J cells before treating with Ex-4 and found that LAMP-2 overexpression can reverse the reduction of cell viability (Fig. 3b) and decrease the percentage of apoptosis cells (Fig. 3c) and accumulation of LC3B-II, p62 and cleaved-caspase-3 (Fig. 3d).

3.4. Ex-4 induced the AR42J cell apoptosis by impairing the autophagic flux

To ascertain whether the imapired autphagic flux was associated with the apoptosis induced by Ex-4, we inhibited the autophagy initiation by pretreating AR42J cells at 0.5 h before exposing in 10 pM Ex-4 with 3-methyladenine (3-MA, 5 mM, Sigma-Aldrich, USA), which reduces the autophagosome formation by inhibiting phosphatidylinositol-3 kinase (PI-3K) signal pathway, and found that pretreatment with 3-MA can reduce the toxicity of Ex-4, which was testified by the increase of cell activity (Fig. 4a), decrease of the percentage of apoptosis cells (Fig. 4b) and downregulation of the cleaved caspase-3 (Fig. 4c).

4. Discussion

We previously found that the pancreatic acinar cells of rats

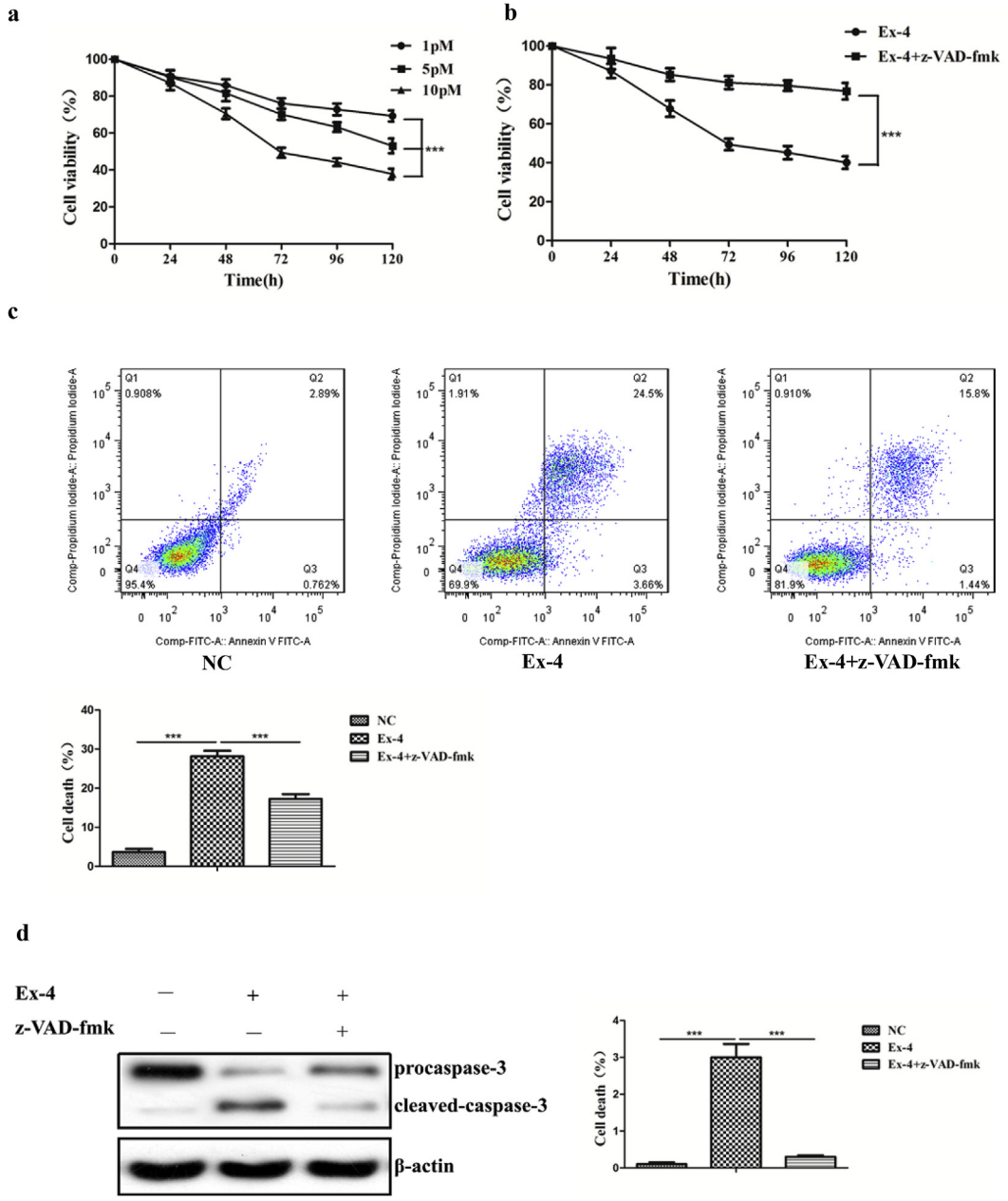

Fig. 1. Ex-4 induced the AR42J cell apoptosis. **a**: The AR42J cells were treated by 1, 5 and 10pM Ex-4 for 24, 48, 72, 96 and 120 h respectively, and Ex-4 significantly inhibited the cell viability in a dose-time dependent manner ($^{***}P < .0001$). **b**: Pretreatment of z-VAD-fmk for 30 min before treatment of 10 pM Ex-4 can notably protect from Ex-4 cytotoxicity ($^{***}P < .0001$). **c**: The AR42J cell apoptosis analysis by flow cytometry demonstrated that Ex-4 can significantly induce the cell apoptosis which can be inhibited by z-VAD-fmk ($^{***}P < .0001$). **d**: The expression of caspase-3 assessed by Western blot also showed that Ex-4 can significantly induce cell apoptosis which can be inhibited by z-VAD-fmk ($^{***}P < .0001$). All experiments were performed in triplicate and the data are presented as mean ± SD.

treated with extended Ex-4 showed pycnosis, the characteristic of apoptosis [9]. In the present study, to further confirm whether Ex-4 can induce pancreatic acinar cells' apoptosis, AR42J cells were treated with various concentrations of Ex-4 respectively for different time. We proved that the Ex-4 treatment decreased the cell viability in dose-time dependent manner which can be alleviated by apoptosis inhibitor v-ZAD-fmk, indicating that Ex-4 does induce cell apoptosis to damage the pancreatic acinar cells. We also identified the percentage of apoptosis cells by flow cytometry and tested the expression of cleaved caspase-3, an essential downstream effector caspase of all kinds of pathways to induce apoptosis [14], which were accordant with the induction of cell apoptosis by the increased percentage of apoptosis cells and upregulated expression of cleaved caspase-3, both which were reversed by apoptosis inhibitor v-ZAD-fmk. Thus, Ex-4 can induce apoptosis in pancreatic acinar cells to damage the pancreatic tissue as shown in our previous results [10]. However, the mechanism that Ex-4 induces pancreatic acinar cells' apoptosis is not clear.

It has been reported that Ex-4 can impair the autophagy flux resulting in the accumulation of vacuoles in the pancreatic acinar cells [10]. Recent studies have shown that impaired autophagy flux can induce cell apoptosis [11–13]. Autophagy flux is a dynamic process in which damaged organelles and cytosolic components are sequestered in double-membrane autophagosomes when cells are stimulated by starvation, stress, some pathophysiological factors and so on. Meanwhile, LC3B-I in the cytoplasm conjugates with

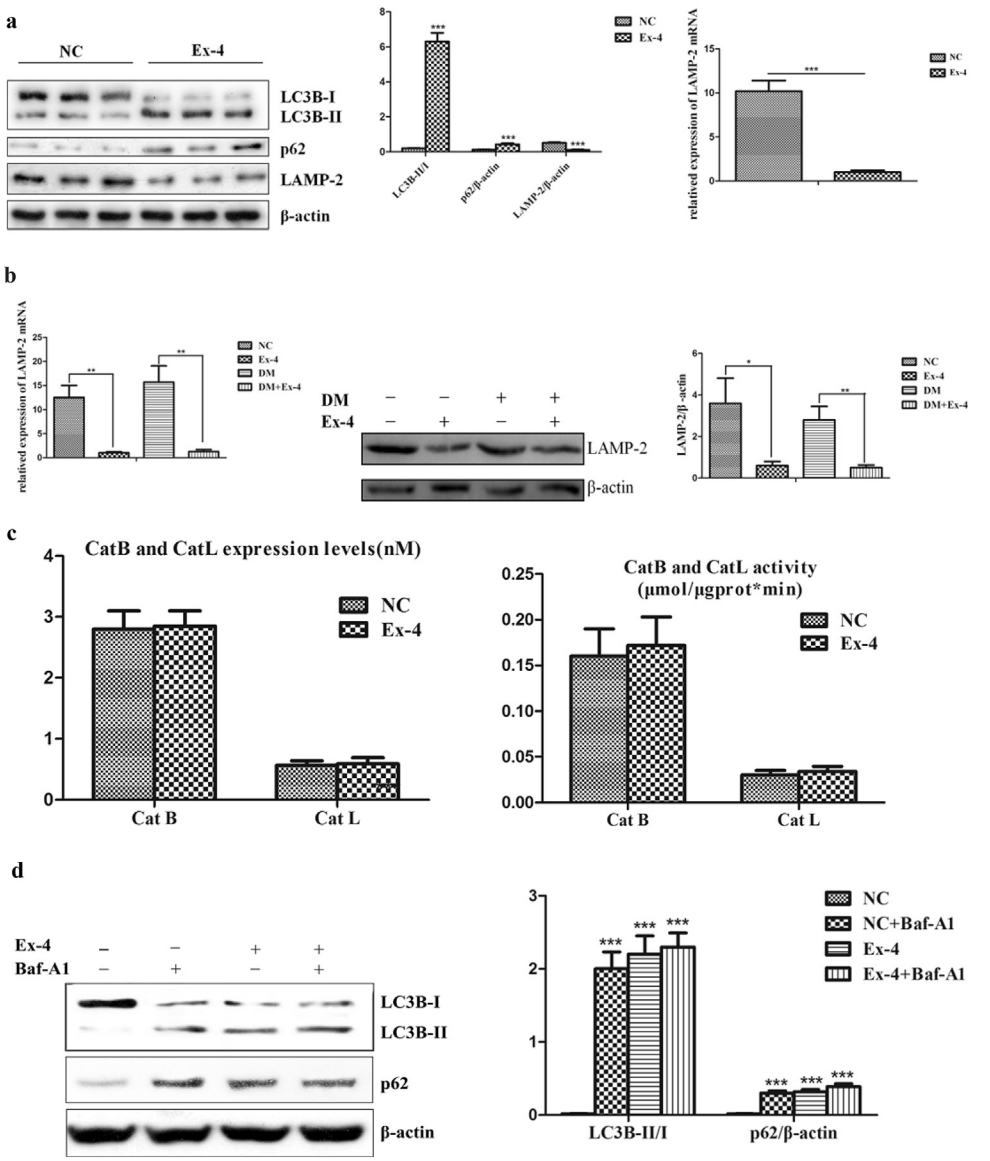

Fig. 2. Ex-4 impaired the autophagic flux by downregulating LAMP-2. **a**: The AR42J cells were treated by 10 pM Ex-4 for 72 h, and it showed that Ex-4 could impair the autophagy flux by increasing the LC3B and p62 protein levels. Meanwhile, Ex-4 notably downregulated the expression of LAMP-2 at mRNA and protein levels ($^{***}P < .0001$). **b:** Exenatide was also found to significantly downregulate the expression of LAMP-2 at mRNA and protein levels in the pancreas tissue of SD rats and diabetes-model rats ($^{*}P < .01$, $^{**}P < .001$). **c:** Ex-4 could not affect the expression or activity of either CatB or CatL. **d**: Pretreatment of autophagy flux inhibitor Baf-A1, which can inhibit the fusion of autophagosome with lysosome, was proved not to aggravate the accumulation of LC3B and p62 induced by Ex-4 ($^{***}P < .0001$).

phosphatidyl ethanolamine on the autophagosomal membrane to form LC3B-II, then autophagosomes are delivered to lysosomes where the autophagic cargo is degraded by lysosomal enzymes, including CatB and CatL [15]. Fusion of autophagosomes with lysosomes to form autolysosomes is depending on LAMP-2 on the membrane of lysosomes [16]. Fusion dysfunction of autophagosome with lysosome resulting from LAMP-2 depletion, or lysosome dysfunction which cannot degrade autophagic cargo will impair the autophagy flux so as to injure the cell [17]. Our results demonstrated that the autophagy marker LC3B-II, which is a quantitative index for monitoring autophagy and formation of autophagosomes, was significantly increased in the AR42J cells after Ex-4 treatment, but p62, which should be degraded by increased autophagy flux, conversely accumulated, indicating that the final step of autophagy signaling was inhibited, persumably by LAMP-2 depletion or lysosome dysfunction. Then we further found that the mRNA and protein expression of LAMP-2 was significantly decreased but the expression and activity of CatB and CatL were not impacted by Ex-4 treatment. Furthermore, inhibition of autophagolysosome formation by Baf-A1, which can inhibit the fusion of autophagosome with lysosome, has no significant impact on impaired autophagic flux. However, upregulation of LAMP-2 by transfecting overexpression plasmid of LAMP-2 can offer protection from Ex-4 toxicity. Taken together, Ex-4 impaired the autophagy flux by downregulating the LAMP-2 to inhibit the fusion of autophagosome with lysosome. However, whether the downregulation of LAMP-2 is directly induced by Ex-4-activated GLP-1 recepter signaling or results from other mechanism is not clear, which deserves our further research and may become a target to prevent the patients treated with Ex-4 from injury of exocrine pancreas.

Diakopoulos et al. [11] have proved that impaired autophagy flux can induce the pancreatic acinat cells' apoptosis and chronic

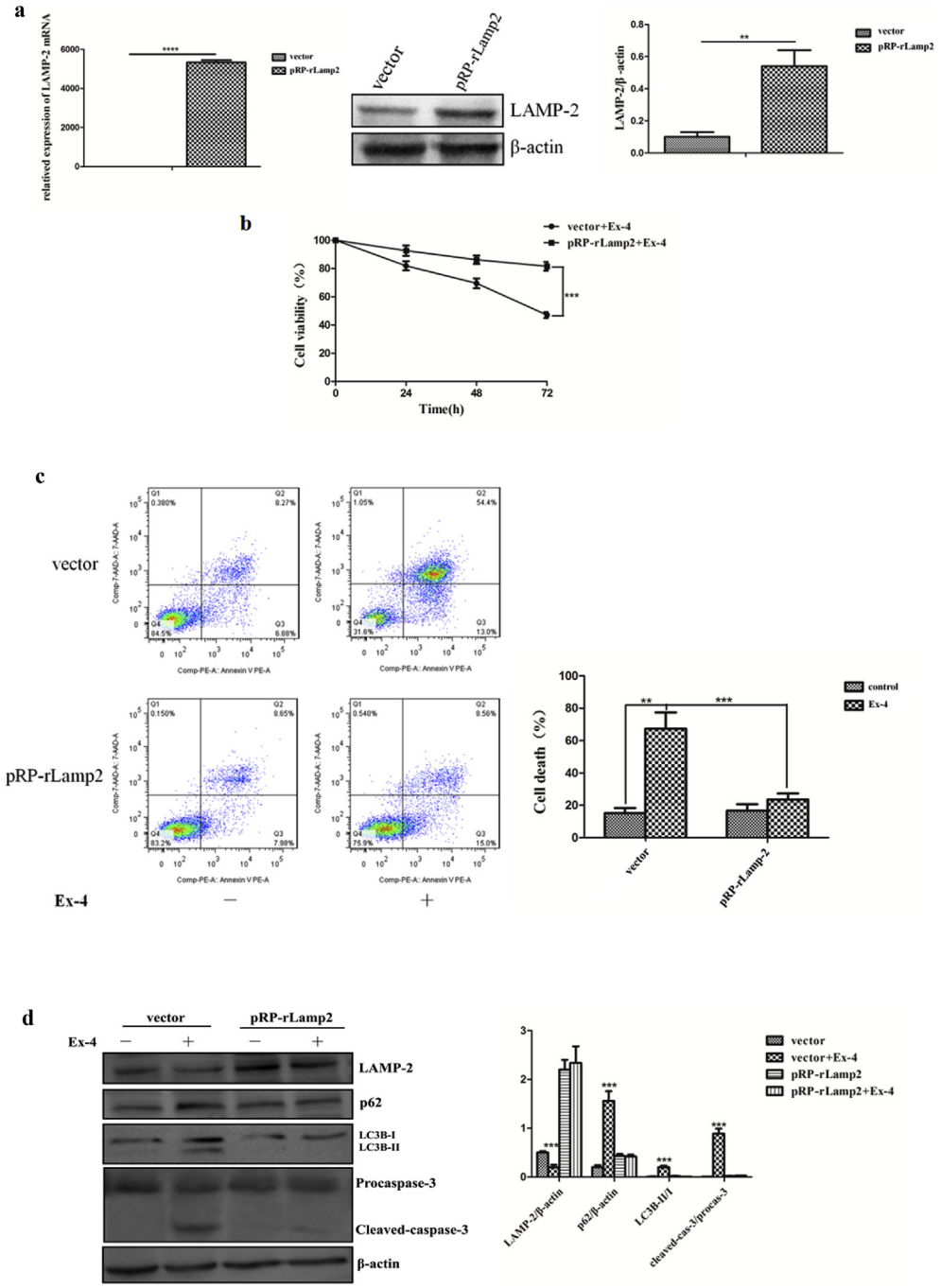

Fig. 3. LAMP-2 overexpression reversed the impaired autophagic flux and apoptosis induced by Ex-4. **a:** pRP-rLamp2 plasmid transfected into the AR42J cells was proved to significantly upregulate the expression of LAMP-2 at the protein level ($^{****}P < .00001$) and mRNA level ($^{**}P < .001$). **b:** Overexpression of LAMP-2 in the AR42J cells by pRP-rLamp2 plasmid transfection was demonstrated to obviously protect from Ex-4 cytotoxicity, shown by MTT assay ($^{***}P < .0001$). **c:** Overexpression of LAMP-2 in the AR42J cells by pRP-rLamp2 plasmid was proved by flow cytometry to decrease the percentage of apoptosis induced by Ex-4 ($^{**}P < .001$, $^{***}P < .0001$). **d:** Overexpression of LAMP-2 in the AR42J cells by pRP-rLamp2 plasmid can decrease the accumulation of LC3B and p62 to alleviate the impaired autophagic flux and can inhibit apoptosis, shown by decreased cleaved-caspase-3 ($^{***}P < .0001$).

atrophic pancreatitis. Sarkar et al. [12] also showed that the accumulation of autophagosomes resulting from impaired autophagy flux can induce cell apoptosis. In the present study, to explore whether pancreatic acinar cell apoptosis results from its impaired autophagy flux, we pretreated AR42J cells with 3-MA to inhibit the initial autophagy flux by blocking the PI-3K signaling pathway, and found that the decrease of cell viability, the cell death and the upregulation of cleaved caspase-3, which were induced by Ex-4, all could be reversed by 3-MA. So the autophagosome accumulation, which occurs when fusion of autophagosome with lysosome is prevented, is a critical step preceding the apoptosis induced by Ex-4.

All the times, we devote ourself to study the mechanism of pancreatic tissue damage induced by Ex-4, which hampered its

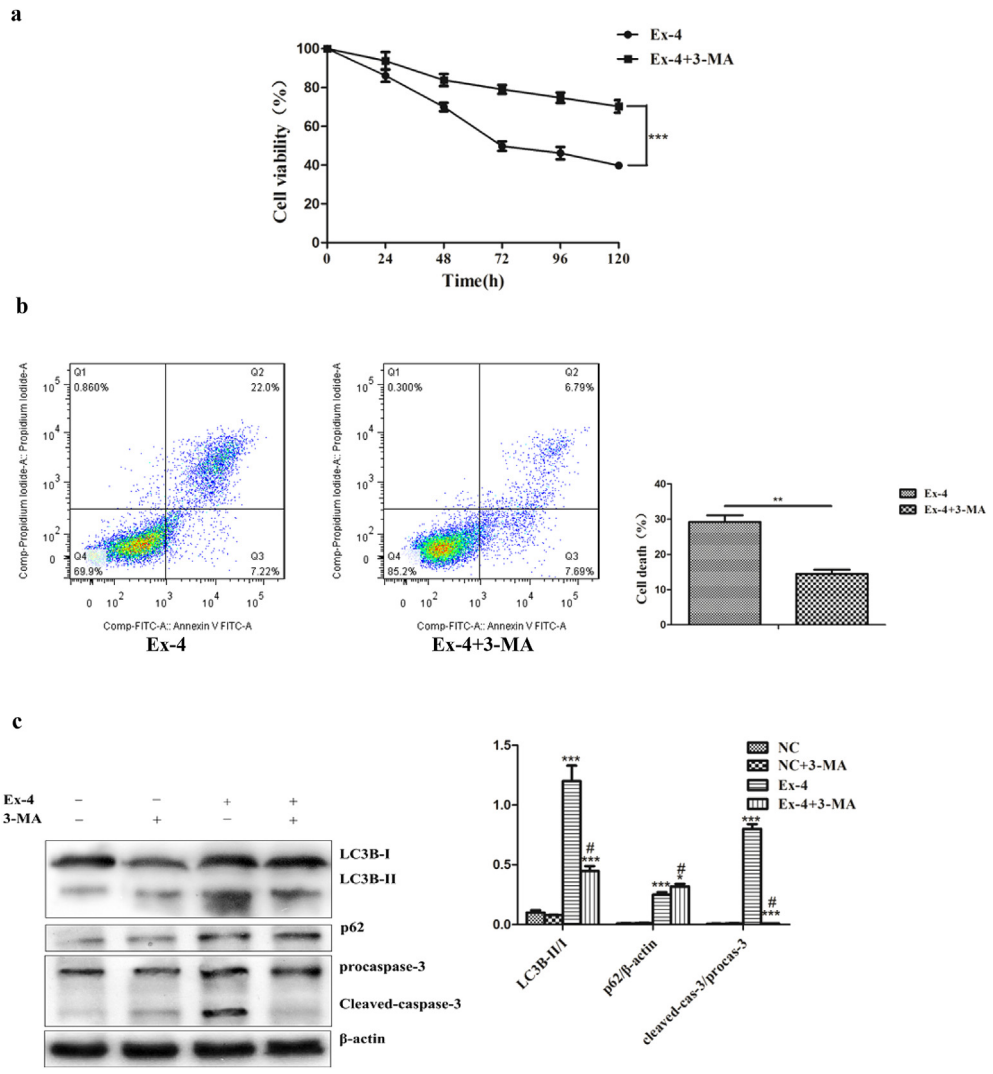

Fig. 4. Ex-4 induced apoptosis by impairing the autophagic flux. **a**: As shown by MTT assay, autophagy initial inhibitor 3-MA can protect from Ex-4 cytotoxicity by recovering the cell viability ($^{***}P < .0001$). **b**: Autophagy initial inhibitor 3-MA was notably found to inhibit the percentage of apoptosis induced by Ex-4 ($^{**}P < .001$). **c**: Pretreatment with 3-MA before Ex-4 can relieve the accumulation of autophagosome, shown by decreased LC3B-II, and significantly inhibit apoptosis, shown by cleaved-caspase-3. # indicates that the difference is significant compared with Ex-4 group ($^{*}P < .01$, $^{***}P < .0001$).

application to treat type 2 diabetes mellitus patients. Although how Ex-4 damages the pancreatic tissue has always been a controversy among reseachers in the recent years, our previous studies have shown that extended Ex-4 treatment induced chronic pancreatitis [8–10]. In this study, we found that Ex-4 can impair autophagy flux to injure the fate of pancreatic acinar cells including the induction of apoptosis, which is the characteristic of chronic pancreatitis. Whether other characteristics of chronic pancreaitis, such as pancreatic fibrosis, are correlated with impaired autophagy flux, is deserving further studies. Some studies have shown that, impaired autophagy flux can induce the cytokine release from pancreatic acinar cells by activating the p62-TRAF6-NFκB pathway [18,19], and certain cytokines may activate the pancreatic stellate cells [20] resulting in pancreatic fibrosis which cannot be directly activated by Ex-4 [21]. Thus, the impaired autophagy flux may be the main mechanism of chronic pancreatitis induced by Ex-4, as found in our previous research. The mechanism of LAMP-2 downregulation in the pancreatic acinar cells treated with Ex-4 will be our next focus of research, and we hope to find a therapeutic target to relieve the damge in exocrine pancreas of patients treated with Ex-4.

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Transparency document

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