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Exenatide Induces Impairment of Autophagy Flux to Damage Rat Pancreas

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Objectives: The study aimed to explore the alteration of autophagy in rat pancreas treated with exenatide.

Methods: Normal Sprague-Dawley rats and diabetes-model rats induced by 2-month high-sugar and high-fat diet and streptozotocin injection were subcutaneously injected with exenatide, respectively, for 10 weeks, with homologous rats treated with saline as control. Meanwhile, AR42J cells, pancreatic acinar cell line, were cultured with exenatide at doses of 5 pM for 3 days. The pancreas was disposed, and several sections were stained with hematoxylin-eosin. Immunohistochemistry was used to measure the expressions of glucagon-like peptide 1 receptor (GLP-1R) and cysteine-aspartic acid protease-3 in rat pancreas, and Western blot was used to test the expressions of GLP-1R, light chain 3B-I and -II, and p62 in rat pancreas and AR42J cells. The data were expressed as mean (standard deviation) and analyzed by unpaired Student's *t*-test.

Results: Exenatide can induce pathological changes in rat pancreas. The GLP-1R, p62, light chain 3B-II, and cysteine-aspartic acid protease-3 in rat pancreas and AR42J cells treated with exenatide were significantly overexpressed.

Conclusions: Exenatide can activate and upregulate its receptor, GLP-1R, then impair autophagy flux and activate apoptosis in the pancreatic acinar cell, thus damaging rat pancreas.

Key Words: exenatide, pancreas, impaired autophagy, apoptosis

(*Pancreas* 2017;46: 83–88)

As an analog of human glucagon-like peptide 1 (GLP-1), which was authorized firstly to treat type 2 diabetes mellitus, exenatide can bind to the GLP-1 receptor (GLP-1R) of pancreatic islet β-cells to promote the secretion of insulin and effectively control the blood glucose level. However, since 2008, when an article published in the *New England Journal of Medicine* reported that exenatide might induce acute pancreatitis,[1] there emerged many reports about this adverse effect, which hampered its application to treat type 2 diabetes mellitus patients.[2–4] Therefore, it is very important to explore the correlation between exenatide and the exocrine pancreas. Our previous studies found that long-term application of exenatide can induce chronic pancreatitis in Sprague-Dawley (SD) rats[5] rather than acute pancreatitis. To explore its mechanism, our group further conducted animal experiments and found that exenatide may activate the pancreatic stellate cell to induce rat pancreatic tissue fibrosis,[6] but Nakamura et al[7] showed that the pancreatic stellate cells treated by GLP-1R agonist cannot be activated in vitro, so the pancreatic stellate cell of rats treated with exenatide may be activated by other factors, such as cytokines released from pancreatic acinar cells (PACs), which may be the main reasons to induce chronic inflammation change of the pancreatic tissue. Gier et al[8] found that exenatide can induce pancreatic ductal cell proliferation and pancreatic intraepithelial neoplasia, which may activate the trypsinogen in the PAC to induce pancreatitis by the obstruction of ductal outflow. The trypsinogen activated in the PAC by impaired autophagy flux, which can induce chronic atrophic pancreatitis in mice shown by Diakopoulos et al,[9] is the initial factor to start the pancreatitis.[10] So, we hypothesized that long-term application of exenatide may induce the impairment of autophagy flux in the PAC, which then damages pancreatic cells to release certain cytokines and thus activates pancreatic stellate cells to induce pancreatic tissue fibrosis. In this study, we preliminarily explored the change of autophagy in the rat pancreatic cell.

MATERIALS AND METHODS

Animals

Animal experimental protocols were approved by the University Animal Care and Use Committee of the Association for Assessment and Accreditation of Laboratory Animal Care. Fifty male SD rats (Hunan Slacjingda Lab Animal Inc, Changsha, China) weighing 180 to 210 g were housed separately in cages in a room with constant temperature and humidity and a 12-hour light-dark cycle throughout the experimental period. Rats were adapted to the environment for 3 days with free access to water and food.

Establishment of the Diabetes-Model Rats

Thirty rats were randomly selected for the diabetes model. After the rats had adapted to the experimental room environment, we measured their fasting plasma glucose levels and took the mean of results as the normal reference value. Then, these rats 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) as previously reported.[6] After 72 hours, we measured the fasting blood glucose level 3 times and took the mean level that was greater than 16.7 mmol/L as the criteria for successful establishment of the diabetes-model rats. Then, we divided the diabetes-model rats into 2 subgroups, namely the diabetes-model exenatide-injected group and the diabetes-model control group. The fasting plasma glucose level of the rats was measured weekly after induction of diabetes to exclude any rats that had glucose levels less than 16.7 mmol/L for 3 times continually from the diabetes-model rat group. During the establishment of the diabetes-model rats, 20 normal SD rats were fed normally and 2 months later were divided into 2 groups, namely the normal control group and the normal exenatide-injected group.

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Received for publication July 5, 2015; accepted June 2, 2016.

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This study was supported by the National Natural Science Foundation of China (Grant No. 81570585) and by the Foundation of Science and Technology Department of Hunan Province (Grant No. 2013FJ6021).

The authors declare no conflict of interest.

DOI: 10.1097/MPA.0000000000000692

Exenatide Treatment and Specimen Collection

After the diabetes-model rats were established, all rats had free access to water and were fed quantitatively. The rats in the normal experimental group and diabetes-model experimental group were injected subcutaneously with exenatide (GL Biochem Shanghai Ltd, Shanghai, China) at a dosage of 5 μg/kg at 8:00 A.M. and 6:00 P.M. of each day, 1 hour before feeding. The rats in the normal control group and the diabetes-model control group were treated with normal saline through subcutaneous injection at an identical volume. All rats were weighed once weekly so that the dosage can be adjusted in a timely manner according to the weight and treated with exenatide or normal saline for 10 weeks. At the end of the experimental period, all rats were injected intraperitoneally with 10% chloral hydrate at a dose of 3 mL per 1 kg (body weight) to anesthetize them. Five rats in each group were chosen randomly to perform thoracotomy on to expose the heart and were perfused with 300 to 450 mL 4% paraformaldehyde solution via the left ventricle until the liver color turned yellow, then the pancreases were harvested and kept in the 4% paraformaldehyde solution. For the other rats of each group, the pancreases were collected directly and each one was divided into 2 pieces, then 1 piece was kept at −80°C and the other kept in the 4% paraformaldehyde solution.

Cell Culture and Treatment

As previously reported,[11] PAC line AR42J cells, purchased from American Type Culture Collection (Manassas, Va), were grown in F-12 K medium with 2 mM L-glutamine, 250 μg/mL amphotericin, 100 units/mL penicillin, 100 μg/mL streptomycin, and 20% fetal bovine serum at 37°C under a humidified condition of 95% air and 5% CO_2. Cells were plated at a density of approximately 10^5 cells/mL in 12-well plates. Morpholino antisense or missense control was added separately to the culture media at 20 μM. Cells were then cultured with exenatide at doses of 5 pM for 3 days before treatment for molecular detection.

Histology

The specimens of pancreas kept in the 4% paraformaldehyde solution were paraffin-embedded and sliced with a thickness of 5 μm, and then the slices were stained by hematoxylin and eosin (HE) and examined under a light microscope.

Immunohistochemical Staining of Cysteine-Aspartic Acid Protease-3 and GLP-1R

The pancreatic tissue from the rats that were perfused with 4% paraformaldehyde solution via the left ventricle were sliced, and immunohistochemistry was carried out using indirect immunoperoxidase detection with primary antibody: rabbit anti–cysteine-aspartic acid protease-3 (caspase-3) polyclonal antibody (19677-1-AP; Proteintech, Chicago, Ill) or anti-GLP-1R antibody (ab39072; Abcam, London, UK). Five no-repeated vision fields under a 400× light microscope were selected on the same section, and the cell with brown granular staining within the cytoplasm was defined as the positive cell, to calculate the number of positive cells in each microscope field and to analyze statistically the mean and standard deviation.

Western Blot

The pancreatic tissues kept at −80°C or cultured AR42J cells were lysed and centrifuged, then the proteins were extracted from the supernatant, separated by electrophoresis, transferred to polyvinylidene difluoride membranes, and probed by antibodies. The antibodies were microtubule-associated protein light chain 3B (LC3B)-specific antibody (18725-1-AP, Proteintech), p62/SQSTM1 antibody (18420-1-AP, Proteintech), anti-GLP-1R antibody (ab39072, Abcam, UK) and β-actin antibody (60008-1-Ig, Proteintech) as loading control. Densitometric analyses were conducted with Quantity One software (BIO-RAD, USA- Hercules, CA) and data were expressed in arbitrary units.

Statistical Analysis

The data were expressed as mean (standard deviation) and were analyzed by unpaired Student *t* test using SPSS 18.0 statistics software (SPSS China, Shanghai, China). A *P* value less than 0.05 was considered to be statistically significant for all tests.

RESULTS

The HE Staining of Pancreatic Tissues

The pancreatic tissue from 9 rats (4 from the normal exenatide-injected group and 5 from the diabetes-model exenatide-injected group) showed pathological changes such as gland structure damage, pancreatic cell atrophy, and cell compartment broadening. The pancreatic tissue in the normal control group and the diabetes-model control group did not show pathological changes (Fig. 1).

The GLP-1R Immunohistochemistry

The GLP-1R–stained cells could not be seen in the exocrine pancreas in the normal control group and diabetes-model control group. But, in the normal exenatide-injected group and diabetes-model exenatide-injected group, GLP-1R staining was seen in the PACs, and the staining was obvious. The numbers of GLP-1R immunohistochemical-positive cells of the pancreatic tissue

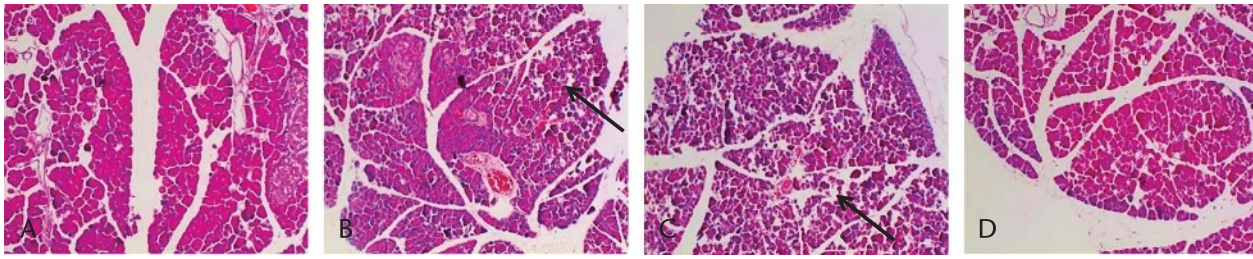

FIGURE 1. Representative images of the pancreatic tissue HE staining in 4 groups. A, Normal control group. The pancreatic tissue showed no lesions. B, Normal exenatide-injected group. The gland structure damage, pancreatic cell atrophy, and cell compartment broadening can be seen clearly (arrow). C, Diabetes-model exenatide-injected group. The pancreatic tissue lesions such as gland structure damage, pancreatic cell atrophy, and cell compartment broadening were apparent (arrow). D, Diabetes-model control group. The pancreatic tissue showed no lesions. Magnification ×100.

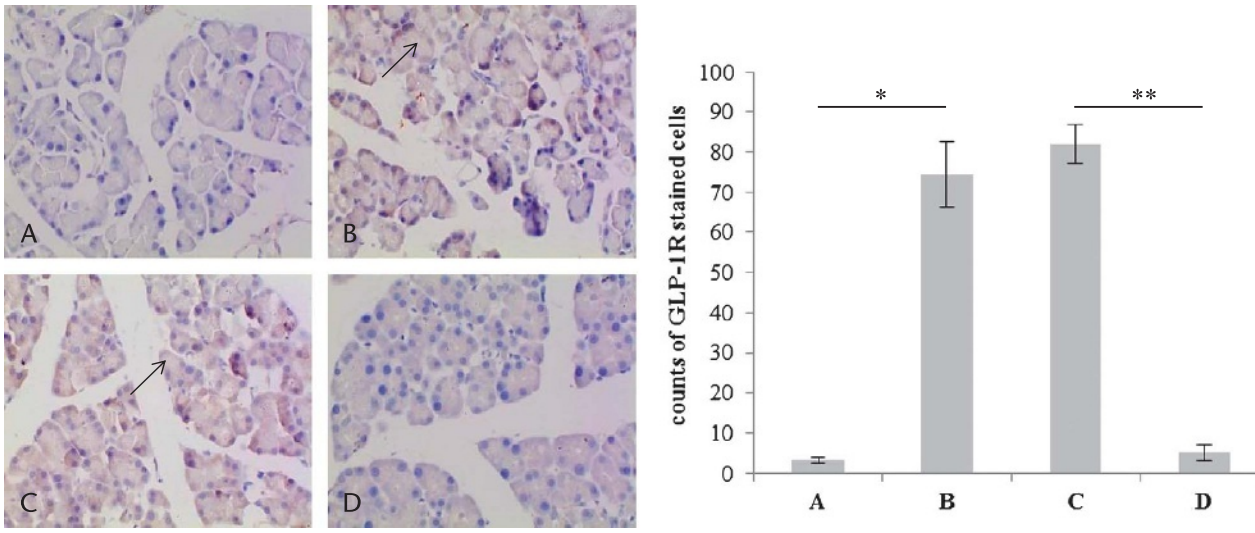

FIGURE 2. Representative photographs of the pancreatic tissue GLP-1R immunohistochemistry (left panel) and statistical evaluation of the counts of GLP-1R–stained cells (right panel). A, Normal control group. The GLP-1R–stained cells (brown) almost could not be seen in the exocrine pancreas. B, Normal exenatide-injected group. The GLP-1R staining was seen in the PACs and the staining was obvious. The counts of GLP-1R–stained cells in this group were higher than the normal control group (*$P = 0.000$). C, Diabetes-model exenatide-injected group. The GLP-1R staining in the PACs was apparent. The counts of GLP-1R–stained cells in this group were higher than the diabetes-model control group (**$P = 0.000$). D, Diabetes-model control group. Stained cells almost could not be seen in the exocrine pancreas. Magnification ×400.

in the 2 exenatide-injected groups were more than their respective control groups, and the differences were statistically significant ($P < 0.05$) (Fig. 2).

The Caspase-3 Immunohistochemistry

Only a few pancreatic cells showed Caspase-3 staining in the normal control group and diabetes-model control group. But, in the 2 exenatide-injected groups, the stained cells appeared more, and the staining was more obvious especially in the diabetes-model exenatide-injected group. The counts of caspase-3 immunohistochemistry-positive cells in the pancreatic tissue of the diabetes-model exenatide-injected group were higher than the ones of the normal exenatide-injected group; the difference was statistically significant ($P < 0.05$) (Fig. 3).

Western Blot Test of Pancreatic Tissue

Western blot showed that the expressions of LC3B-II, p62, and GLP-1R in rat pancreatic tissues of the normal exenatide-injected group and the diabetes-model exenatide-injected group were higher, and the LC3B-I protein level in rat pancreatic tissues of the 2 exenatide-injected groups was lower than their respective control groups. Through densitometric analysis, the LC3B-II level

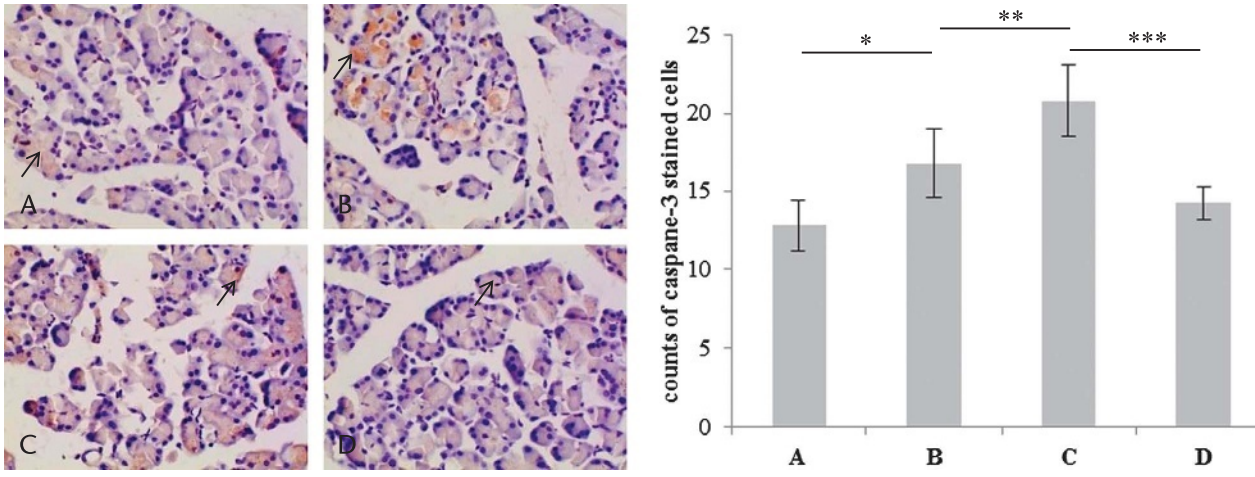

FIGURE 3. Representative photographs of the pancreatic tissue caspase-3 immunohistochemistry (left panel) and statistical evaluation of the counts of caspase-3–stained cells (right panel). A, Normal control group. Immunohistochemistry showed a few caspase-3–stained cells (arrow) and the caspase-3 staining was thin. B, Normal exenatide-injected group. Caspase-3 staining was seen in the PACs and the staining was obvious. The counts of caspase-3–stained cells (arrow) in this group were higher than the normal control group (*$P = 0.011$). C, Diabetes-model exenatide-injected group. The caspase-3 staining in the PACs was apparent. The counts of caspase-3–stained cells (arrow) in this group were higher than the diabetes-model control group and the normal exenatide-injected group (**$P = 0.001$, C vs D; ***$P = 0.022$, C vs B). D, Diabetes-model control group. A few caspase-3–stained cells (arrow) could be seen in the exocrine pancreas. Magnification ×400.

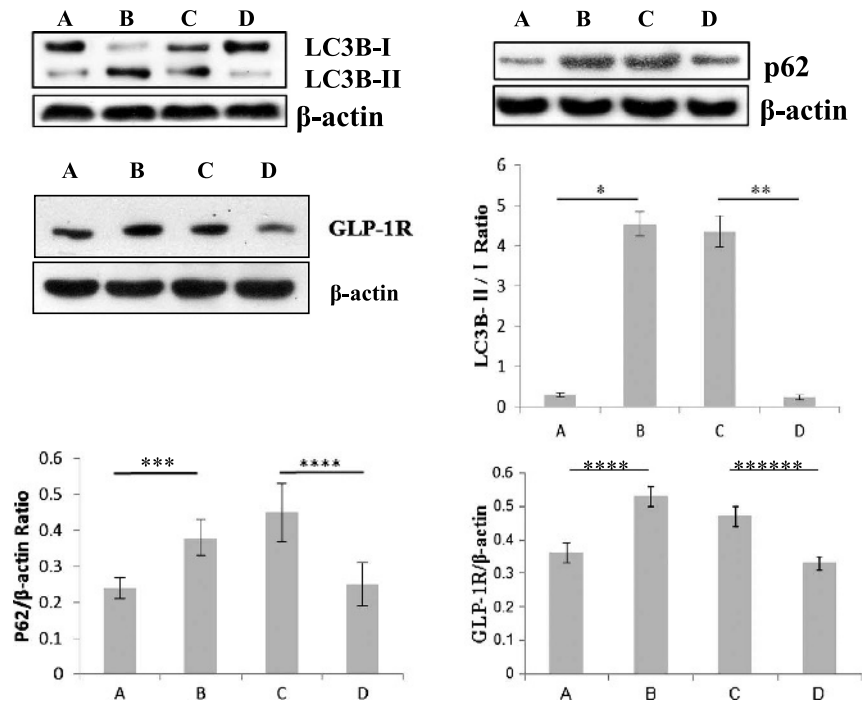

FIGURE 4. The protein levels of LC3B-I, LC3B-II, p62, and GLP-1R in pancreatic tissues of SD rats by Western blot method and the densitometric analysis of LC3B-II level relative to LC3B-I, p62 and GLP-1R level relative to β-actin. A. Normal control group. The expression of LC3B-I was higher than LC3B-II level. B. Normal exenatide-injected group. The expression of LC3B-II was higher than LC3B-I level, and the expression of p62 and GLP-1R in pancreatic tissues of this group was higher than the normal control group. Densitometric analysis showed that the ratios of LC3B-II to LC3B-I, p62 to β-actin, and GLP-1R to β-actin in this group were higher than the normal control group (*P = 0.000, ***P = 0.013, *****P = 0.002 respectively). C. Diabetes-model exenatide-injected group. The expression of LC3B-II was higher than LC3B-I, and the expression of p62 and GLP-1R in pancreatic tissues of this group was higher than the diabetes-model control group. Densitometric analysis showed that the ratios of LC3B-II to LC3B-I, p62 to β-actin, and GLP-1R to β-actin in this group were higher than the diabetes-model control group (**P = 0.000, ****P = 0.025, respectively). D. Diabetes-model control group. The expression of LC3B-I was higher than LC3B-II level.

relative to LC3B-I, p62 level relative to β-actin, and GLP-1R level relative to β-actin were higher in pancreatic tissue of rats treated with exenatide than the control (Fig. 4).

Western Blot Test of PAC

Western blot in PACs showed the same results as the rat pancreatic tissue: that the expressions of p62, LC3B-II, and GLP-1R in the pancreatic cell treated with exenatide were higher. Through densitometric analysis, the LC3B-II level relative to LC3B-I, the p62 level relative to β-actin and GLP-1R level relative to β-actin were higher in PAC treated with exenatide than the control (Fig. 5).

DISCUSSION

In this study, after being treated with exenatide, pathological changes occurred in the pancreatic tissue of 4 normal SD rats and 5 diabetes-model rats such as gland structure damage, pancreatic cell atrophy, and cell compartment broadening, which conformed with our previous experimental findings. Our previous experiments using the scanning electron microscope found that the change of pycnosis was characteristic of apoptosis in PACs of rats treated with exenatide. To know whether apoptosis is activated in pancreatic cells of rats administered by exenatide, we used immunohistochemistry to test the expression of caspase-3, which is an essential downstream-effector caspase for all kinds of pathways to induce apoptosis,[12] and found that the expression of caspase-3 was upregulated in the pancreatic tissue of normal rats and diabetes-model rats with long-term treatment of exenatide. Thus, the long-term treatment of exenatide may induce pancreatic cells to apoptosis and atrophy, which leads to chronic injury of the pancreatic tissue. However, the way that exenatide induces pancreatic cells to apoptosis is not still clear.

Exenatide is a kind of GLP-1R agonist and plays its biologic role through combining with GLP-1R of cells. In the normal pancreatic tissue of rats, GLP-1R expression was detected mainly in pancreatic β-cells and was not detected in PACs, the expression of GLP-1R in the PACs was upregulated in the pancreas of humans and rats with chronic pancreatitis,[7] and exenatide can promote apoptosis of human pancreatic cancer cells by activating GLP-1R.[13] In this study, we found that GLP-1R expression was also present in the PACs of rats treated with exenatide, which means that exenatide may upregulate the expression of GLP-1R in the PACs and induce PACs to apoptosis by combining with the GLP-1R of PACs.

Diakopoulos et al[9] found that impaired autophagy flux can induce the PACs to apoptosis and chronic atrophic pancreatitis. This study aimed to detect the expressions of autophagic markers LC3B and p62 to show the change of autophagy in pancreatic cells. The LC3B, the mammalian homolog of yeast Atg8, is involved in autophagosome formation during autophagy. In immunoblotting, LC3B is detected as 2 bands: the one above represents LC3B-I, which mainly exists in the cytoplasm when autophagy is not activated, and the other one below is LC3B-II, which is conjugated with phosphatidyl ethanolamine and is located in the autophagosomal membrane.[9] When the cell is stimulated by starvation, stress, some pathophysiological factors, and so on, autophagy will be induced in the cytoplasm and many autophagosomes

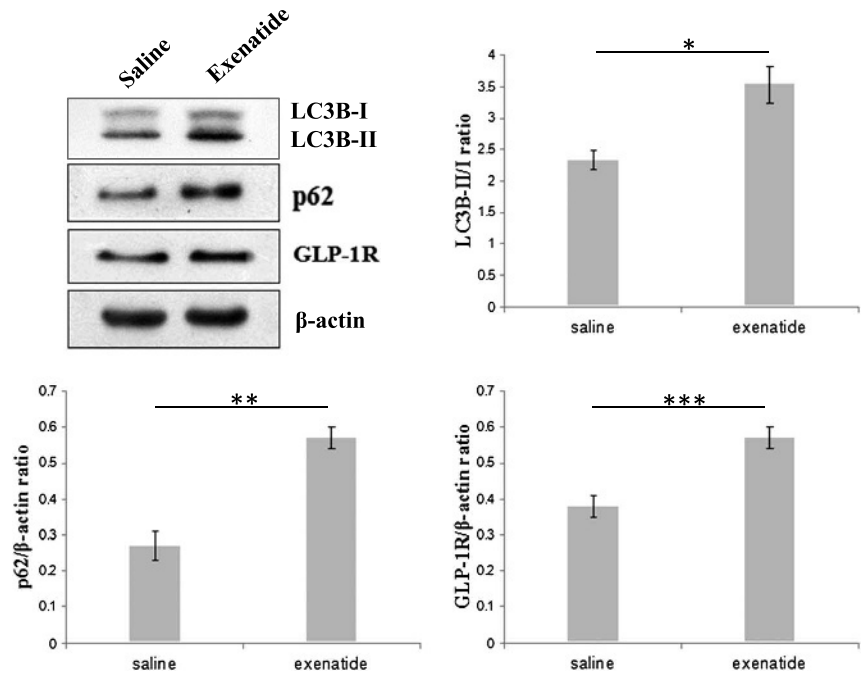

FIGURE 5. The expressional levels of LC3B-I, LC3B-II, p62, and GLP-1R in PACs by Western blot methods and the densitometric analysis of LC3B-II level relative to LC3B-I, p62, and GLP-1R levels relative to β-actin. The ratio of LC3B-II to LC3B-I in the PACs treated by exenatide was higher than the saline group (*$P = 0.004$), which means that exenatide can activate autophagy in the PACs. However, the expression of p62 protein (which is degraded by the autophagic flux) in the PAC of exenatide group was higher than the saline group (**$P = 0.000$). The expression of GLP-1R (with which exenatide binds to play its activity) in the PAC treated with exenatide also was higher than the saline group (***$P = 0.001$).

are formed; meanwhile, LC3B-I in the cytoplasm will conjugate with phosphatidyl ethanolamine on the autophagosomal membrane to form LC3B-II, which is present on the autophagosomal membrane, then the autophagosome will fuse with a lysosome for degradation of the sequestered materials. The processing 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 of the cell. Examining the cytosolic LC3B-II/I ratio is a quantitative method for monitoring autophagy in the cell, and the LC3B-II/I ratio will increase when autophagy is induced.[14,15]

An alternative method for monitoring the autophagic flux is measuring p62 degradation.[16] The p62 is a kind of a ubiquitin-binding protein, which can recognize and bind with ubiquitinated proteins in protein aggregates, damaged mitochondria, and peroxisomes, shut them into autophagosomes by binding LC3B-II, and then degrade them by lysosomal enzymes.[9] Thus, the expression level of p62 could be an indicator of autophagic degradation, and the accumulation of p62 can indicate the autophagy suppression or inhibition of autophagic degradation.[17,18]

In this study, the LC3B-II/I ratio and the expression level of p62 in the rat pancreatic tissue of the exenatide-injected group and diabetes-model exenatide-injected group were significantly higher than their respective control groups, but there were no significant differences between the 2 exenatide-injected groups and the 2 control groups. Thus, long-term treatment of exenatide may induce autophagy, but induced autophagy cannot degrade p62 adequately, which meant that autophagy flux was impaired. To verify it, the PACs were cultured with exenatide at doses of 5 pM for 3 days, and similar results were gained. Impaired autophagy flux can lead to accumulation of autophagosomes or autolysosomes and vacuolization of cells, which conformed to our previous experimental findings that the rat PAC vacuoles increased in the exenatide-injected group. Impaired autophagy flux cannot fully degrade certain cytoplasmic constituents, which accumulate to damage the cell. Diakopoulos et al[9] found that the accumulation of p62 could promote apoptosis by activating the Nrf2/Nqo1/p53 signaling pathway. So, impaired autophagy flux may be the mechanism of pancreatic cell apoptosis and tissue atrophy, which deserves further research. Some researchers found that impaired autophagy can lead to cytokine release from the PAC by activating the p62-TRAF6-NFkB pathway,[19,20] and certain cytokines can activate the pancreatic stellate cells to drive pancreatic fibrosis.[21] These can interpret our previous findings that long-term administration of exenatide may lead to the activation of the pancreatic stellate cells, which cannot be directly activated by exenatide.[7] Thus, the impaired autophagy flux may be the main mechanism of pancreatic tissue pathological changes found in our research. However, the mechanism of how the binding of exenatide with the GLP-1R of PACs induces the impaired autophagy flux is not clear, which deserves further study to elaborate. And, the reason of the autophagy flux impairement in the PAC treated with exenatide will be next focus of research to get a target to prevent the pancreas of patients treated with exenatide from being damaged.

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