



Original article

GLP-1R agonist may activate pancreatic stellate cells to induce rat pancreatic tissue lesion

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ABSTRACT

Objective: To explore the mechanism of GLP-1R agonist-induced rat pancreatic tissue lesion.**Methods:** Thirty SD male rats were divided into three groups, namely GLP-1R agonist experimental group, diabetes-model experimental group and control group. Diabetes-model rats were induced by streptozotocin and high-sugar high-fat diet. GLP-1R agonist group and diabetes-model group were administered with GLP-1R agonist in dose 5 μg/kg each time, twice a day. After 10 weeks of treatment, the amount of matrix metalloproteinase (MMP)-2 and MMP-9, and expression of α-smooth muscle actin (α-SMA) and type III collagen protein in pancreatic tissue were measured.**Results:** The amount of MMP-2 and MMP-9 in GLP-1R agonist group and diabetes-model group were significantly higher than the control group. Compared with the GLP-1R agonist group, the diabetic model group had more severe pathological changes of pancreatic tissue interstitial edema, inflammatory cell infiltration, glandular atrophy and fibrosis, and significantly increased pancreatic tissue MMP-2 and MMP-9 levels, significantly increased α-SMA and collagen III-positive cell counts, all the differences were statistically significant. α-SMA and type III collagen were expressed in all parts of the lesions of GLP-1R agonist group and diabetes-model group. α-SMA can only be observed in the vessel wall in control group, however, in the other two groups, α-SMA can also be observed in pancreatic acinar cell interstitia, in addition to vessel wall.**Conclusions:** Long-term subcutaneous GLP-1R agonist injection may activate pancreatic stellate cells, causing the expression of α-SMA and collagen III and the amount of MMP-2 and MMP-9 in pancreatic acinar cell interstitial significantly increasing, and thus inducing chronic inflammatory change.

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Glucagon-like peptide-1 receptor (GLP-1R) agonists (exenatide) is the first incretin agonist used in clinical treatment of diabetes, but shortly after its clinical use, there emerged report that it might induce acute pancreatitis. Ahmad and Swann (2008) [1] first published a paper on this, since then similar reports have increased [2–5]. The preliminary animal experiments by our group [6] showed that long-term application of exenatide can lead to part of the SD rat pancreatic tissue with chronic inflammatory changes. The results of clinical studies also indicated that exenatide may induce acute pancreatitis [3]. Although relative reports continue to emerge, the mechanism of GLP-1R agonist-induced inflammatory lesions of the pancreas is rarely reported. Since the roles of the

glucagon-like peptide-1 (GLP-1) confirmed as far can not explain the relationship between GLP-1R agonists and pancreatic stellate cell activation, we hypothesized that there may also exist other activation pathways of pancreatic stellate cells, in addition to the classic pathways such as ethanol, transforming growth factors and inflammatory cytokines. This study sought to further explore the possible mechanisms of GLP-1R agonist-induced inflammatory lesions of pancreatic tissue from the histopathology and immunohistochemistry aspects.

1. Materials and methods

1.1. Materials

Exenatide (purity 98%): Shanghai Peptide Biotechnology Inc., China; ELISA (Enzyme-linked immunosorbent assay) kits for MMP-2 and -9 (matrix metalloproteinase-2, -9): R&D Inc., USA; α-smooth

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muscle actin (α -SMA) and type III collagen immunohistochemistry kits: Santa Cruz Biotechnology, Inc., USA; Streptozotocin (STZ), Hematoxylin & Eosin (HE): Sigma Inc., USA.

1.2. Animal grouping and feeding

Animal experimental protocols were approved by the University Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Thirty Sprague–Dawley male rats (Hunan Slac Jingda Lab Animal Inc., Changsha, China) with the weight of 280–310 g were divided into three groups according to the principle of complete random design, 10 rats for each group, namely GLP-1R agonist experimental group, diabetes-model experimental group and control group. Rats had free access to water, injected subcutaneously of saline or GLP-1R agonists (exenatide), at 8:00 am and 6:00 pm daily, 1 h after quantitative feeding.

1.3. Establishment of rat diabetes model

The fasting plasma glucose level was measured in SD rats for a week, and the results (mean) was taken as the normal reference value. Then rats were fed with high-sugar high-fat diet for two months. 72 h after intraperitoneal injection of streptozotocin (STZ, 35 mg/kg), fasting blood glucose level was measured three times, the mean level increasing (greater than 16.7 mmol/L) was taken as the successful modeling criteria for the diabetic rats.

1.4. Drug delivery methods

GLP-1R agonists (exenatide) was injected subcutaneously to the GLP-1R agonist experimental group and diabetes model experimental group with 5 μ g/kg each time, 2 times/day, at 8:00 am and 6:00 pm, 1 h before feeding. Rats were weighted once weekly and GLP-1R agonist (exenatide) dosage was adjusted according to the weight. The blank control group was treated with normal saline subcutaneous injection for a period of 10 weeks.

1.5. Specimen collection

After the experimental period of 10 weeks, all rats were anesthetized with 10% chloral hydrate, 0.3 ml per 100 g body weight, by intraperitoneal injection. Thoracotomy was taken to expose the heart, the rat was perfused via left ventricle by 250–300 ml 0.01 M PBS buffer, followed by 300–450 ml 4% poly-formaldehyde solution, until the rat liver color turned yellow, and the pancreatic tissue was collected.

1.6. Pancreatic tissue MMP-2 and MMP-9 detection

Collected pancreatic tissue was immediately placed in 4% paraformaldehyde solution and fixed in 4 °C refrigerator for 24 h, and then placed in 0.2% tissue preservation solution (sodium azide solution). MMP-2 and MMP-9 were detected for all collected specimens by the enzyme-linked immunosorbent assay (ELISA) kit

according to the instructions. Pancreas tissue (5 mg) was homogenated and centrifuged, and the supernatant was taken out and diluted 1:10, incubated for twice, added with tetracarboxylic benzidine (TMB), the substrate for horseradish peroxidase (HRP), and the TMB optical density at 450 nm for each titration plate was measured, using the samples provided by the kit to make MMP-9 standard linear regression curve and finally to obtain the actual concentration of MMP-9. All specimens were tested in pair and the average was calculated. The pancreatic tissue MMP-2 levels were detected using the same method.

1.7. Histopathological examination of pancreatic tissue

Collected pancreatic tissue was immediately placed in 4% formaldehyde solution at 4 °C for 24 h. After fixation, the tissue was carried through routine dehydration, transparency, wax dipping, embedment, wax block making, and slicing (thickness of 5 μ m). The slices were stained with HE and examined under microscope.

1.8. Immunohistochemical determination of α -SMA and type III collagen proteins in pancreatic tissue

Pancreatic tissue slices were treated by grilling, dewaxing, hydration, antigen retrieval, blocking, adding primary antibody, washing, blocking, adding secondary antibody, washing and chromogenation. Under Olympus BX-51 light microscope at 400 \times magnification, five unrepeated visionary fields were selected, to select cells with brown granular staining within the cytoplasm as the positive cells, to calculate the number of positive cells, and the mean and standard deviation were statistically analyzed.

1.9. Statistics

SPSS 17.0 statistics software was used for data analysis. Measured data were expressed as mean \pm standard deviation. MMP-2 and MMP-9 ELISA results, and α -SMA and type III collagen protein immunohistochemistry positive cell counts, were analyzed by two-sample T testing, and the test standard was $\alpha = 0.05$.

2. Results

2.1. Pancreatic tissue MMP-2 and MMP-9 expressions

Compared with the control group, the MMP-2 and MMP-9 expressions in pancreatic tissue of the GLP-1R agonists experimental group and the diabetes model experimental group were significantly higher, the difference was statistically significant ($P < 0.05$) (Table 1).

2.2. Pancreas tissue pathology

The pancreatic tissue HE staining results are shown in Fig. 1. In the GLP-1R agonist experimental group and diabetes model experimental group, there appeared some pancreatic tissue lesions, such as partial acinar cell edema, acinar interstitia expansion, and

Table 1
Pancreatic tissue MMP-2 and MMP-9 expressions, and α -SMA and type III collagen protein-positive cell counts ($\bar{X} \pm S$).

Group	MMP-2 (μ g/L)	MMP-9 (μ g/L)	α -SMA cell counts	Type III collagen cell counts
Blank control group ($n = 10$)	186.98 \pm 23.24	49.37 \pm 7.08	13.4 \pm 5.97	10.6 \pm 4.93
GLP-1R agonist experimental group ($n = 10$)	306.07 \pm 59.82 ^a	67.24 \pm 14.73 ^a	29.5 \pm 8.80 ^a	29.3 \pm 12.95 ^a
Diabetes model experimental group ($n = 10$)	365.08 \pm 89.55 ^a	87.37 \pm 13.39 ^a	79.3 \pm 27.23 ^{a,b}	56.0 \pm 27.21 ^{a,b}

^a $P < 0.05$ vs. blank control group.

^b $P < 0.05$ vs. GLP-1R agonist experimental group.

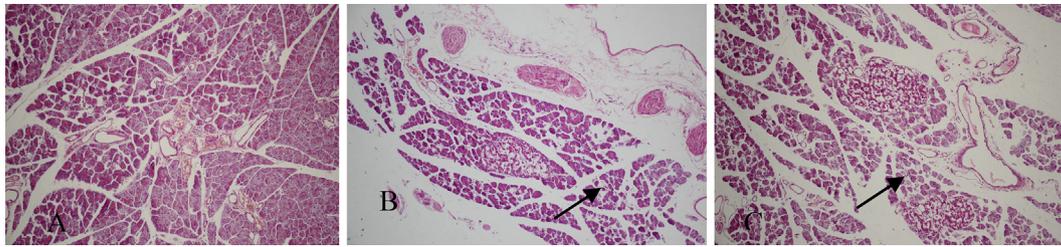


Fig. 1. The pancreatic tissue HE staining results. A. Control group; B. GLP-1R agonist experimental group. Partial acinar cell edema and acinar interstitia expansion can be seen (arrow). the pancreatic tissue lesion. C: Diabetes model experimental group. Partial acinar cell edema and acinar interstitia expansion are apparent (arrow). Magnification: $\times 100$.

chronic inflammatory changes. These changes were particularly obvious in two cases of the GLP-1R agonist experimental group. The degrees of acinar cell edema and acinar interstitia expansion of the diabetic model experimental group are significantly heavier than the GLP-1R agonist experimental group, indicating that the chronic inflammatory changes are greater in the diabetic model experimental group.

2.3. Pancreas tissue immunohistochemistry

2.3.1. α -SMA

Pancreatic tissue α -SMA immunohistochemistry results are shown in Fig. 2. Whereas the α -SMA staining was found only in the vascular wall in the blank control group, α -SMA was also seen around the pancreatic acinar cells and interstitial in the GLP-1R agonist experimental group and diabetes model experimental group. Compared with the control group, the stained cells appeared more and staining are more obvious in the GLP-1R agonist experimental group and diabetic model experimental group. The positive staining area of the diabetic model experimental group was greater than the GLP-1R agonist experimental group, indicating the pancreatic tissue lesion had greater extent in the diabetes model experimental group.

2.3.2. Type III collagen

The pancreatic tissue collagen type III immunohistochemical results are shown in Fig. 3. Compared with the control group, the stained cells appeared more and staining are more obvious in the GLP-1R agonist experimental group and diabetic model experimental group. The positive staining area of the diabetic model experimental group was greater than the GLP-1R agonist experimental group, indicating the pancreatic tissue lesion had greater extent in the diabetes model experimental group.

2.3.3. Pancreatic tissue α -SAM and type III collagen immunohistochemistry positive cell counts

Compared with the control group, α -SMA and collagen type III protein immunohistochemical positive cells of pancreatic tissue in

the GLP-1R agonist experimental group and diabetes model experimental group increased significantly, the difference was statistically significant ($P < 0.05$). The counts of α -SMA and type III collagen immunostaining positive cells in the pancreatic tissue of the diabetic model experimental group were significantly higher than those of the GLP-1R agonist experimental group, the difference was statistically significant ($P < 0.05$) (Table 1).

3. Discussion

In this study, pancreatic tissue HE staining showed that the GLP-1R agonist experimental group had typical chronic pancreatitis changes, including interstitial edema, inflammatory cell infiltration, glandular atrophy and fibrosis, especially obvious in two cases. For the blank control group, above pathological changes were not observed in the pancreatic tissue, further confirming our previous experimental findings [6]. In this study, new experimental group (diabetes model experimental group) exhibited chronic pancreatitis formation, and the severity of interstitial edema, inflammatory cell infiltration, glandular atrophy and fibrosis was heavier than the GLP-1R agonist experimental group. Four cases of the diabetes model experimental group were especially obvious. That the severity of the diabetic model experimental group was heavier than the GLP-1R agonist experimental group may have two reasons. (1) GLP-1R agonists may activate the pancreatic stellate cells (PSCs) in the islets. Homo-Delarche et al. [7] have shown that there exist PSCs in the pancreatic islets of diabetic patients. Therefore, using the same dose of GLP-1R agonist in different pancreatic tissue may affect more PSCs in the diabetic model experimental group, causing more extensive and serious damage. (2) Although the diabetes pathogenesis is not yet clear, under the long-term diabetes pathophysiological state, the PSCs in the pancreatic tissue may increase the sensitivity to certain drugs such as GLP-1R agonist, more likely to be activated to cause chronic inflammatory disease. The above two reasons may exist alone or in parallel, but they still need further basic and clinical research to confirm. Matrix metalloproteinase (MMP) belongs to the zinc-dependent endopeptidase family, and its most common function is the degradation of the

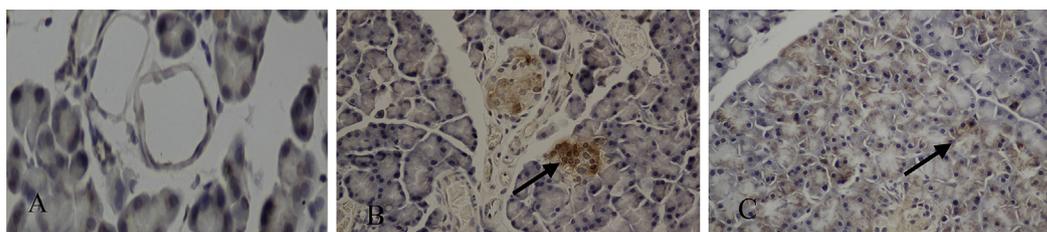


Fig. 2. Pancreatic tissue α -SMA immunohistochemistry results. A: Blank control group. Pancreatic tissue α -SMA stained cells (brown) are few, staining was not obvious and distributed only around the vessel wall. B: GLP-1R agonist experimental group. α -SMA staining was obvious. In addition to the vessel wall, the staining was also seen around the pancreatic acina and interstitia (arrows). C: diabetes model experimental group. α -SMA stained cells are popular and the staining was obvious. In addition to the vascular wall, the staining can also be found in the pancreatic acina and interstitia (arrows). Magnification: $\times 400$.

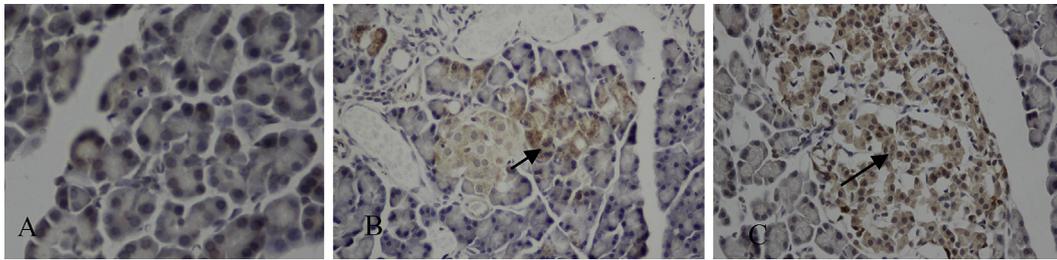


Fig. 3. Pancreatic tissue type III collagen immunohistochemistry results. A: Blank control group. Pancreatic tissue type III collagen stained cells are few. B: GLP-1R agonist experimental group. The type III collagen stained cells are more and the staining is obvious (arrows). C: Diabetes model experimental group. The type III collagen staining cells are popular and the staining is obvious (arrows). Magnification: $\times 400$.

extracellular matrix (ECM). Under physiological conditions, the ECM in the tissue is always in the dynamic balance of synthesis and degradation. The disorder of this dynamic balance is related to the occurrence of many diseases. Two enzyme systems among a variety of regulation systems, MMPs and tissue inhibitor of metalloproteinases (TIMPs), play a critical role in the synthesis and degradation of ECM. PSC activation is the initiating factor of the pancreas chronic inflammatory disease, playing a key role in the pathophysiological process of pancreatic fibrosis. Upon activation, PSCs secrete ECMs, such as collagen, elastin, laminin, etc. Haber et al. [8] have revealed that α -SMA is a marker of PSC activation and its expression is increased in a variety of chronic inflammatory disease. Our present study showed that α -SMA positive cell counts of the GLP-1R agonist experimental group and the diabetic model experimental group were greater than the control group, the degree of staining was deeper, and the difference was statistically significant, indicating that the PSCs in the pancreatic tissue of these two experimental groups may be activated. Our experiment also found that the type III collagen-positive cell counts of GLP-1R agonist experimental group and diabetes model experimental group were greater than the control group, the degree of cell staining was also deeper than the control group, and the difference was statistically significant, further confirming that the PSCs in the pancreatic tissue of these two experimental groups may be activated. Apte et al. [9] found that activated PSCs can secrete large amounts of MMP to participate in the degradation of extracellular matrix. In this study, pancreatic tissue ELISA results showed that MMP-2 and MMP-9 expressions in the pancreatic tissue of the GLP-1R agonist experimental group and the diabetes model experimental group were higher than the blank control group, the difference was statistically significant, indicating that there is a correlation between the activation of PSC and the increased MMP-2 and MMP-9 expression, suggesting that the increase of MMP-2 and MMP-9 expression may depend on the activation of PSCs. That the increase of MMP-2 and MMP-9 expression in the diabetic model experimental group was more obvious than that in the GLP-1R agonist experimental group also confirmed that the pancreatic tissue of the diabetic experimental group may have more PSC activation.

Chronic pancreatitis is the most common risk factor for pancreatic cancer, so we pay more attention to the GLP-1R agonist-induced chronic pancreatitis. Pancreatic cancer is one of the common clinically refractory tumors, its early diagnosis rate being very low, often already at advanced stage when diagnosed, and the 5-year survival rate does not exceed 10% [10,11]. Vakkila et al. [12] have shown that the risk of chronic pancreatitis patients to suffer from pancreatic cancer was significantly increased (15–16 times)

than the general population. By inference, the long-term use of GLP-1R agonists will increase the pancreatic cancer risk. Although GLP-1R agonists have many advantages on the treatment of type 2 diabetes, its risk to increase pancreatic cancer should not be overlooked anymore. This study shows that long-term application of GLP-1R agonist may activate PSCs and then cause chronic inflammatory disease of the pancreatic tissue, but its mechanism of activating PSCs is not yet clear and further study is needed to elaborate it.

Disclosure

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