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# RFamide-related peptide-3 promotes alpha TC1 clone 6 cell survival likely via GPR147

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is closely related to islet alpha cell mass and viability. Several types of RFamide-related peptides (RFRPs) are involved in regulating proliferation and function of islet cells. However, current understanding of the role of RFamide-related peptide-3 (RFRP-3) in pancreatic alpha cells is limited. Therefore, we investigated the expression of the RFRP-3 receptor, G protein-coupled receptor 147 (GPR147), in mouse islets and alpha TC1 clone 6 cells, and evaluated the function of RFRP-3 on alpha cells. We show that GPR147 is expressed in mouse islets and alpha cell lines. In addition, RFRP-3 promotes survival of alpha cells under conditions of hyperglycemia and serum starvation. Mechanistic evidence demonstrates that RFRP-3 activated PI3K/AKT and ERK1/2 signaling cascades and treatment with an antagonist of GPR147, 1-adamantanecarbonyl-Arg-Phe-$NH_2$ (RF9) blocked this function. These findings indicate a novel effect of RFRP-3 in promoting alpha cell survival, likely via GPR147.

## 1. Introduction

Type 2 diabetes mellitus (T2DM) is a rapidly growing chronic disease affecting millions of individuals worldwide [1]. T2DM patients are characterized by increased circulating levels of glucagon [2,3]. Glucagon is the main hyperglycemic hormone that is produced by pancreatic alpha cells [4]. Alpha cell mass and viability play crucial roles in glucose homeostasis. In pancreatic islets, alpha cells comprise roughly 20% of the total cell population [5], and alpha cell expansion has been reported in T2DM patients [6]. Therefore, studying the mechanisms and molecules related to alpha cell mass and viability is of utmost importance.

RFamide-related peptides (RFRPs), which possess an arginine-phenylalanine-amide (Arg-Phe-$NH_2$) motif at their C-terminus, have been discovered in the past few decades. To date, five members of the RFamide-related peptide superfamily have been reported [7]. Gonadotropin inhibitory hormone (GnIH) is one of these members and is also known as RFRP-3 in mammals. RFRP-3 has been identified in the hypothalamus in rats, monkeys, and humans [8–10], and its cognate receptors are G protein-coupled receptors, GPR147, and GPR74 [11]. Studies have shown that RFRP-3 binds to GPR147 with high affinity and that the affinity of RFRP-3 for GPR74 is lower [12–14].

A previous study has shown that RFRP-3 negatively regulates the function of the gonadotrophin-releasing hormone (GnRH) neuron and plays a role in regulating reproductive function [15]. In addition, RFRP-3 has been demonstrated to impart stimulatory effects on food intake [16,17]. Furthermore, in metabolic deficiency, the expression of the *RFRP* gene is higher [18]. Studies on RFRP-3 and its receptor signaling pathway have indicated that these are highly active in organs that are related to regulating metabolic homeostasis [18]. In addition, other members of RFRPs, 43RFa and 26RFa, promote the survival of pancreatic beta cells and islets [19]. Based on the above findings, we hypothesized that GPR147 may be present in islets. In consideration of pancreatic alpha cell functions in perceiving metabolic changes and maintaining whole body fuel homeostasis, we were particularly interested in investigating the expression of GPR147 in mouse islets and alpha cells, as well as the underlying effects of RFRP-3 on alpha cells.

*Abbreviations:* T2DM, type 2 diabetes mellitus; RFRPs, RFamide-related peptides; RFRP-3, RFamide-related peptide-3; GPR147, G protein-coupled receptor 147; GPR74, G protein-coupled receptor 74; RF9, 1-adamantanecarbonyl-Arg-Phe-$NH_2$; GnIH, gonadotropin inhibitory hormone; GnRH, gonadotrophin releasing hormone; IP, intraperitoneal; FISH, fluorescent *in situ* hybridization; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hank's buffered salt solution; SF, serum-free; PI3K, phosphatidylinositol-3-kinase; ERK1/2, extracellular signal-regulated kinase; PCNA, proliferating cell nuclear antigen

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## 2. Materials and methods

### 2.1. Cell culture and reagents

Alpha TC1 clone 6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, UT, USA), containing 10% fetal bovine serum (FBS; HyClone, Tauranga, New Zealand), 1 g/L glucose, and 1% penicillin/streptomycin (HyClone, UT, USA). Cells were grown in an incubator at 37 °C and 5% $CO_2$. Prior to experiments, cells were cultured in the presence of 25 mM glucose for 72 h, and after rinsing with serum-free medium, cultured in serum-free medium for an additional 12 h. Then, cells were incubated without or with RFRP-3 for 24 h. In the present study, a GPR147 antagonist RF9 (catalog No. G-4800.0005, Bachem, Switzerland) and rat RFRP-3 (catalog No. H-5846.0001, Bachem, Switzerland) were used. Antibodies directed against AKT, P-AKT, ERK1/2, P-ERK1/2 and PCNA were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibody against β-actin and PD-98059 were obtained from Sigma-Aldrich (St. Louis, MO, USA). MK-2206 was from MedChemExpress (Monmouth Junction, NJ, USA). The primary antibody against GAPDH was obtained from CW Biotech (Beijing, China). The primary antibody against GPR147 was obtained from Biorbyt (Cambridge, United Kingdom). The primary antibody against glucagon was obtained from Abcam (Cambridge, United Kingdom).

### 2.2. Animal and islet isolation

Adult male C57BL/6 mice aged 12–14 weeks and weighing 25–30 g were purchased from the Experimental Animal Center of Forth Military Medical University (Xian, China). Prior to the experiment, mice were acclimatized for one week under a 12-h light/dark cycle with free access to water and food. All animal experiments were approved by the Animal Care and Use Committee of Forth Military Medical University. A total of 10 mice were used.

Mice were anesthetized by intraperitoneal (IP) injection of diluted pentobarbital sodium (60 mg/kg). Pancreatic islets were isolated after infusion of collagenase (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) into the pancreas. The pancreata were placed in a 38 °C water-bath, incubated for 13–17 min, and gently shaken every 3 min. Then, the digested pancreata were centrifuged at 1000 rpm for 5 min (4 °C) with cold polysucrose/sodium diatrizoate solution (Sigma-Aldrich, St. Louis, MO, USA) to purify the islets. For subsequent protein extraction, the islets were manually selected under a microscope and transferred to a 15-mL tube, containing 5 mL of Hank's buffered salt solution (HBSS; HyClone, UT, USA).

### 2.3. RT-PCR and real-time PCR

Total RNA was extracted using TRIzol reagent (Wuhan Servicebio Technology, Ltd., Wuhan, China). RNA extraction and purification were performed following the manufacturer's instructions. RNA concentration and purity were determined by UV-spectroscopic (NanoDrop2000, Thermo Fisher Scientific, Waltham, MA, USA) analysis, and the D260/D280 ratio was between 1.8-2.0. Subsequently, 2 μg of total RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's guidelines. Next, 2 μL of cDNA template was amplified in triplicate on a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster, CA, USA) using a FastStart Universal SYBR Green Master (Rox) kit (Roche Life Science, Indianapolis IN, USA) following the manufacturer's guidelines. The reaction conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The primer sequences used for real-time PCR were as follows: GPR147 Sense: ATG AGC GGC TTG GTA CAG G; GPR147 Anti Sense: CGG AAA GGG TGT ACG ATG CA; GAPDH Sense: CCT CGT CCC GTA GAC AAA ATG; GAPDH Anti Sense: TGA GGT CAA TGA AGG GGT CGT. Mouse GAPDH was used as an internal control. Amplicons (98 bp for GPR147, 133 bp for GAPDH) were separated by 1.5% agarose gel electrophoresis and were subsequently tested by ethidium bromide staining.

### 2.4. Fluorescent in situ hybridization (FISH) combined with immunofluorescence

Alpha TC1 clone 6 cells were fixed in 4% paraformaldehyde at room temperature for 20 min. Slides then underwent enzymatic digestion at 37 °C for 25 min. Subsequently, the slides were incubated with primary antibody against glucagon (dilution 1: 200, Abcam) at 4 °C overnight and were then washed with phosphate-buffered saline (PBS) and incubated with a Cy3-conjugated secondary antibody rabbit anti-mouse for 1 h at room temperature. After prehybridization, slides were incubated with the probe-containing hybridization solution (8 ng/mL, FAM-labelled probe) at 37 °C overnight. Probe sequences of GPR147 were as follows: 5′-FAM-GGGTA GGAGC GGTTG CGAGC ATCCA G-FAM-3′. Subsequently, slides were washed by washing buffer and were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Wuhan Servicebio Technology Ltd., Wuhan, China). Images were captured using an ECLIPSE CI upright microscope (Nikon Corporation, Tokyo, Japan) at the indicated magnifications.

### 2.5. Western blot analyses

Alpha TC1 clone 6 cells and islets were washed three times with cold PBS, then cell lysis buffer including 1 mM of phenylmethylsulfonyl fluoride (Wuhan Boster Biological Technology, Ltd., Wuhan, China), protease inhibitors (Wuhan Boster Biological Technology, Ltd., Wuhan, China), and phosphatase inhibitors (Thermo scientific, Rockford, IL, USA) were added. Equal amounts of protein (15 μg) were separated using 10% SDS-PAGE and were transferred onto PVDF membranes (Millipore, Burlington, MA, USA). Membranes were blocked with 5% skim milk and were incubated with primary antibodies directed against ERK 1/2, P-ERK 1/2, AKT and PCNA (dilution 1:1,000, Cell Signaling Technology), P-AKT (dilution 1:2,000, Cell Signaling Technology), GPR147 (dilution 1: 400, Biorbyt), β-actin (dilution 1: 2000, Sigma-Aldrich), and GAPDH (dilution 1:1,000, CW biotech). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Wuhan Boster Biological Technology Ltd., Wuhan, China), protein bands were visualized using a ChemiDoc XRS (Bio-Rad, CA, USA). Densitometric analysis was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA). GAPDH or β-actin was used as the internal control for normalization.

### 2.6. Immunohistochemistry

Mouse pancreata were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were dewaxed and rehydrated with alcohol and phosphate buffer. Alpha TC1 clone 6 cells were fixed in 4% paraformaldehyde at room temperature for 20 min. After heat-induced antigen retrieval and removal of endogenous peroxidase, slides were blocked with 3% BSA (30 min). Next, the slides were incubated with antibody against GPR 147 (1:200 dilution) for 12 h at 4 °C. Slides were washed and incubated with HRP-labeled goat anti-rabbit antibody (1:200 dilution) for 1 h at room temperature. After incubation with 3,3-diaminobenzidine (DAB), the slides were counterstained with hematoxylin. Slides incubated without the antibody against GPR 147 were used as a negative control.

### 2.7. Cell survival

Cell survival was assessed by Cell Counting Kit-8 (Dojindo Laboratories, Japan). Briefly, alpha TC1 clone 6 cells were seeded in 96-well plates at $5 \times 10^3$ cells/well in DMEM complete growth medium. The next day, the medium was replaced with medium containing