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DPP-4 inhibitor des-F-sitagliptin treatment increased insulin exocytosis from *db*/*db* mice β cells

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ABSTRACT

Incretin promotes insulin secretion acutely. Recently, orally-administered DPP-4 inhibitors represent a new class of anti-hyperglycemic agents. Indeed, inhibitors of dipeptidyl peptidase-IV (DPP-4), sitagliptin, has just begun to be widely used as therapeutics for type 2 diabetes. However, the effects of sitagliptin-treatment on insulin exocytosis from single β-cells are yet unknown. We therefore investigated how sitagliptin-treatment in *db*/*db* mice affects insulin exocytosis by treating *db*/*db* mice with des-F-sitagliptin for 2 weeks. Perfusion studies showed that 2 weeks-sitagliptin treatment potentiated insulin secretion. We then analyzed insulin granule motion and SNARE protein, syntaxin 1, by TIRF imaging system. TIRF imaging of insulin exocytosis showed the increased number of docked insulin granules and increased fusion events from them during first-phase release. In accord with insulin exocytosis data, des-F-sitagliptin-treatment increased the number of syntaxin 1 clusters on the plasma membrane. Thus, our data demonstrated that 2-weeks des-F-sitagliptin-treatment increased the fusion events of insulin granules, probably via increased number of docked insulin granules and that of syntaxin 1 clusters.

1. Introduction

Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are known to act as incretins. GIP, a 42-amino acids hormone secreted from K cells of the upper small intestine [1,2], acts directly on pancreatic islets to stimulate insulin secretion [3,4]. GLP-1, a 31-amino acid hormone produced from proglucagon and secreted from L cells of the lower intestine and colon [5], also acts on islets and stimulates insulin secretion in isolated islets [6]. Although long-term intravenous infusion of GLP-1 has been shown to improve glycemic control [7,8], rapid proteolytic degradation of incretins catalyzed by DPP-4 [9,10] hampered the therapeutic use of incretins for type 2 diabetes. Recently, orally-administered DPP-4 inhibitors represent a new class of antihyperglycemic agents reflecting their ability to extend the biological effects of incretins [8,11,12]. DPP-4 is an enzyme expressed on the endothelial lining of the vasculature and on the cell surface of a variety of organs [13]. DPP-4 degrades and inactivates the incretin hormones such as GLP and GIP [13,14]. Thus, DPP-4 inhibitors decrease both postprandial and fasting plasma glucose levels in patients with type 2 diabetes, by increasing action forms of incretin hormones.

Multiple DPP-4 inhibitors were characterized in preclinical and clinical studies [15–18], and the prolonged biological effects of incretin hormones by DPP-4 inhibitors were assessed on pancreatic β-cell mass and function [19], however, it is not clear whether the prolonged effect acts on insulin exocytosis from β cells. Recent clinical data showed that DPP-4 inhibitors are more efficient in Japanese type diabetic patients [20–23], probably because reduced early insulin secretory capacity could be partly due to that considerably lower levels of intact GLP-1 in Japanese subjects [24]. Thus, it is very important to evaluate the effects of DPP-4 inhibitors on insulin exocytosis. In the present study, we administered DPP-4 inhibitors, des-F-sitagliptin, to *db*/*db* mice for 2 weeks, then analyzed the insulin exocytosis, behavior of single insulin granule motion, by TIRF imaging system.

2. Materials and methods

2.1. Animals

Diabetic male *db*/*db* mice were obtained from a commercial breeder (Japan CREA, Tokyo, Japan). Mice were housed under a 12-h light/dark cycle and given free access to food and water until the start of experiments, which were conducted with 6 week-old mice. The *db*/*db* mice were divided into two treatment groups.

Abbreviations: DPP-4, dipeptidyl peptidase-IV; CCD, charge-coupled device; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; KRB, Krebs ringer buffer; TIRF, total internal reflection fluorescence; TIRFM, TIRF microscopy; t-SNARE, target membrane soluble NSF attachment protein receptor.

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Des-fluoro-sitagliptin was administered orally by premixing with regular chow at 1.1% (wt/wt) for 2 weeks in the treatment group according to previous report [25]. The mixing and repelleting were performed by research diet (NJ, USA). Body weights were measured in all animals at the end of the treatment period.Blood samples were collected from tail veins, and plasma glucose was measured by GlutestR (Sanwa Kagaku Kenkyusho) during the study.

2.2. Chemicals

Des-F-sitagliptin, a des-fluoro analog of sitagliptin [19] was provided from Merck & Co., Inc. (Whitehouse Station, NJ, USA).

2.3. TIRF microscopy (TIRFM)

At the end of 6 week treatments, pancreatic islets of Langerhans were isolated by collagenase digestion according to Nagamatsu et al. [26], with some modifications. Isolated islets were dissociated into single cells by incubation in Ca^{2+}-free Krebs Ringer buffer (KRB) containing 1 mM EGTA, and cultured on fibronectin-coated (KOKEN Co. Ltd., Tokyo, Japan) high-refractive-index glass (Olympus) in RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 200 U/ml penicillin, and 200 μg/ml streptomycin as described [27]. Cells were maintained at 37 °C in an atmosphere of 5% CO_2. To label the insulin secretory granules, pancreatic β cells were infected with the recombinant adenovirus Adex1CA insulin-GFP [27]. The Olympus total internal reflection system was used with a high-aperture objective lens (Apo 100× OHR; NA 1.65, Olympus). To observe GFP and Alexa Fluor 488, we used a 488 nm laser line for excitation and a 515 nm long-pass filter for the barrier. Images were then projected onto a cooled charge-coupled device (CCD) camera (DV887DCSBV, ANDOR) operated with Metamorph version 6.3 (Universal Imaging). Images were acquired at 300 ms intervals. The space constant for the exponential decay of the evanescent field was approximately 43 nm. For real-time images of GFP-tagged insulin granule motion, β cells were placed on the high refractive index glass, mounted in an open chamber, and incubated for 30 min at 37 °C in Krebs Ringer buffer (KRB) containing 110 mM NaCl, 4.4 mM KCl, 1.45 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.3 mM calcium gluconate, 4.8 mM $NaHCO_3$, 2.2 mM glucose, 10 mM HEPES (pH 7.4), and 0.3% BSA. Cells were then transferred to the thermostat-controlled stage (37 °C) of TIRFM and stimulation with glucose was achieved by addition of 52 mM glucose-KRB into the chamber (final 22 mM glucose). Most analyses, including tracking (single projection of differing images) and area calculations were performed using Metamorph software. To analyze the data, fusion events were manually selected, and the average fluorescence intensity of individual granules in a 1 μm × 1 μm square placed over the granule center was calculated. The number of fusion events was manually counted while looping about 5000 frame time-lapses.

2.4. Perfusion experiments in mouse pancreas

Overnight fasted mice, which were treated with and/or without des-F-sitagliptin for 2 weeks, were used in perfusion experiments as previously described [28] with minor modifications. The perfusion was begun with a 15-min equilibration period with KRB buffer containing 2.8 mM glucose. The glucose concentration of the perfusate was shifted from 2.8 to 16.7 mM glucose during a 40 min-period. Fractions were collected at 1 ml/min, and insulin in aliquots of perfusate was measured by an insulin ELISA kit (Morinaga).

2.5. Oral glucose tolerance test (OGTT) and plasma GLP-1

OGTT was performed after 2 weeks treatment as described previously [27]. Mice were fasted for over night, and the tests were carried out at 9:30 AM. Blood samples were drawn from the tail vein at 0, 30, 60, and 120 min after glucose administration (0.8 g glucose/kg body weight). Plasma GLP-1 was measured by a GLP-1 ELISA kit (Shibayagi) at 0 and 30 min after glucose administration.

2.6. Immunohistochemical analysis

For TIRF analysis of syntaxin 1A and SNAP-25 clusters, and docked insulin granules at the plasma membrane, pancreatic β cells were fixed, permeabilized with 2% paraformaldehyde and 0.1% Triton X-100, and processed for immunocytochemistry as described previously [29]. Cells were labeled with monoclonal anti-insulin antibodies (Sigma–Aldrich), anti-HPC1-antibodies (Sigma–Aldrich), and anti-SNAP-25 antibodies (Wako, Co. Ltd. Osaka, Japan), then processed with Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes). Immunofluorescence staining was detected by TIRFM. For morphometric analysis of islets, paraffin-embedded pancreas sections (10 μm) were labeled with anti-insulin antibody and detected by an avidin–biotin-peroxidase technique (Vector Laboratories). Sections were collected at 500 μm intervals from tissue blocks, and all islets in the sections were analyzed as islet area over total pancreatic area. Images were acquired using Olympus microscope IX70, equipped with a CCD camera, and analyzed using Metamorph software (Universal Imaging).

2.7. Statistical analysis

Results are means ± SEM from at least three different experiments performed independently. Statistical analysis was performed by analysis of variance (ANOVA) followed by Dunnett's test or Tukey–Kramer's test using the Statview software for Windows version 5.0 (SAS Institute, Cary, NC).

3. Results

In vivo data showed that there was no significant difference of fasting blood glucose levels between des-F-sitagliptin-treated and -untreated *db*/*db* mice (data not shown), though, the oral glucose tolerance test demonstrated that des-F-sitaglilptin-treatment significantly improved the impaired glucose tolerance observed in *db*/*db* mice (Fig. 1). We next examined how 2-weeks administration of des-F-sitagliptin affect plasma GLP-1 levels in *db*/*db* mice. After des-F-sitagliptin-treated *db*/*db* mice were challenged by glucose at 0.8 g/kg, serum was collected at 0, and 30 min, then GLP-1 was assayed by ELISA. As shown in Fig. 2, des-F-sitagliptin administration increased plasma GLP-1 levels when challenged by glucose, but there was no significant difference in basal status.

To examine the time-course of insulin secretory response to high-glucose, pancreas perfusion experiments were performed using 2-week-treated *db*/*db* mice. As shown in Fig. 3A, 16.7 mM glucose elicited insulin secretion was potentiated by sitagliptin-treated *db*/*db* mice. In order to evaluate the sitagliptin effect in detail on glucose-evoked insulin exocytosis, the dynamic motion of individual insulin granules tagged with GFP was observed under TIRF microscopy. As previously reported [27], fusion of insulin granules during 1st phase release mainly involved previously docked granules, whereas fusion from newcomer granules was observed during 2nd phase release. As shown in Fig. 3B, the pattern of the dynamic motion of insulin granules was not changed between sitagliptin-treated and -untreated cells, though, the number of fusion events during 1st phase were increased in sitagliptin-treated β