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Quantitative visualization of synchronized insulin secretion from 3D-cultured cells

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ABSTRACT

Quantitative visualization of synchronized insulin secretion was performed in an isolated rat pancreatic islet and a spheroid of rat pancreatic beta cell line using a method of video-rate bioluminescence imaging. Video-rate images of insulin secretion from 3D-cultured cells were obtained by expressing the fusion protein of insulin and *Gaussia* luciferase (Insulin-GLase). A subclonal rat INS-1E cell line stably expressing Insulin-GLase, named iGL, was established and a cluster of iGL cells showed oscillatory insulin secretion that was completely synchronized in response to high glucose. Furthermore, we demonstrated the effect of an antidiabetic drug, glibenclamide, on synchronized insulin secretion from 2D- and 3D-cultured iGL cells. The amount of secreted Insulin-GLase from iGL cells was also determined by a luminometer. Thus, our bioluminescence imaging method could generally be used for investigating protein secretion from living 3D-cultured cells. In addition, iGL cell line would be valuable for evaluating antidiabetic drugs.

1. Introduction

The communication networks in animal tissues are achieved by cell-to-cell and cell-to-extracellular matrix (ECM) interactions along with inter- and intra-cellular signals. Three-dimensional (3D) cell culture systems, which exhibit the similar physiological properties to tissues, could be used to investigate these communication networks [1].

In pancreas, insulin is secreted in a pulsatile manner from the beta cells of the islets into the blood. The main extracellular signal for increasing insulin secretion is an increase in the concentration of glucose in the blood. It is known that a decrease and irregular oscillations in insulin secretion cause type 2 diabetes [2], but the molecular mechanisms of oscillatory insulin secretion are not fully understood. Previously, we successfully visualized glucose-stimulated insulin secretion from the conventional two-dimensional (2D) cultured pancreatic beta cell line using a high-speed electron-multiplying charge-coupled device (EM-CCD) camera [3]. The fused protein of insulin to *Gaussia* luciferase (Insulin-GLase) as a reporter was transiently expressed and the luminescence signals from cells were detected as a marker of insulin secretion [3]. However, no method of visualizing proteins secreted from 3D-cultured cells was established, and there are no reports on direct observation of the dynamics of synchronous insulin secretion in a pancreatic islet.

In this report, we describe synchronous insulin secretion from an isolated rat islet by the method of quantitative bioluminescence imaging in 3D-cultured cells. We have also established a cell line, named iGL, which is a subclone of INS-1E expressing Insulin-GLase, and demonstrate the effects of an antidiabetic drug on insulin secretion from an islet-like spheroid of iGL cells.

2. Materials and methods

2.1. Plasmid vectors for secretory expression of Insulin-GLase

The fused protein of insulin to GLase (Insulin-GLase) was used as a reporter protein to monitor insulin secretion, as previously described [3]. To obtain a stable transformant expressing Insulin-GLase from the rat pancreatic beta cell line INS-1E [4], a vector of pcDNA3-hINS-GLuc containing the gene encoding for human pre-proinsulin fused to the amino-terminus of GLase was used [3].

Abbreviations: GLase, *Gaussia* luciferase; Insulin-GLase, the fused protein of insulin to *Gaussia* luciferase; iGL, a subclonal rat INS-1E cell line stably expressing Insulin-GLase; ECM, extracellular matrix; 3D, three-dimensional; 2D, two-dimensional.

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To express Insulin-GLase in rat pancreatic islets, an adenoviral vector of pAd-hINS-GLuc was prepared using ViraPower Adenoviral Expression System (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA) as follows. The cDNA fragment coding for the pre-proinsulin fused to GLase was obtained from pcDNA3-hINS-GLuc by the PCR amplification (25 cycles, 15 s at 96 °C, 15 s at 55 °C, 60 s at 68 °C) using KOD-Plus ver. 2 DNA polymerase (Toyobo, Osaka, Japan) with a primer set of hINS-P3 (5′ CACC GGATCC A GCCACC **ATG** GCC CTG TGG ATG CGC CT 3′, 4 bases for directional TOPO cloning underlined and methionine codon bolded) and GL4 (5′ CCC TCT AGA **TTA** GTC ACC ACC GGC CCC CTT 3′, stop codon bolded). The PCR-amplified DNA fragment was recombinated to a pENTR/D-TOPO vector (Invitrogen) to give a pENTR-hINS-GLuc. Further, the cDNA fragment of Insulin-GLase in pENTR-hINS-GLuc was recombinated to pAd-V5-DEST (Invitrogen) using LR Clonase (Invitrogen) to produce pAd-hINS-GLuc.

2.2. Preparation of the adenovirus solution for expressing Insulin-GLase in rat pancreatic islets

Recombinant adenovirus for expressing Insulin-GLase in rat pancreatic islets was prepared according to the manufacturer's instruction. Briefly, pAd-hINS-GLuc was digested with the restriction enzyme of *Pac*I (Thermo Fisher Scientific) and transfected to HEK293A cells (Invitrogen) with FuGENE HD (Promega, Madison, WI). HEK293A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Wako Pure Chemicals, Osaka, Japan, Cat. No. 044-29765) containing 10% FBS (HyClone, Logan, UT). The transfected cells were cultured for 7 days, and cell extracts were used as the source of adenovirus for infection to rat pancreatic islets. The titer of the adenoviral solution was determined by plaque numbers formed in HEK293A cell monolayer on an agarose/DMEM solution in a 6-well plate (Falcon, Cat. No. 353046, Corning, NY) for 14 days and stained with thiazolyl blue tetrazolium bromide (Sigma, St. Louis, MO). The titer was estimated to be 8×10^8 pfu/mL.

2.3. Isolation of rat pancreatic islets and Insulin-GLase expression with the adenoviral vector

Pancreatic islets were isolated from 7 to 10-weeks old Wistar rats by treatment with collagenase. Krebs-Ringer bicarbonate Hepes buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24.2 mM $NaHCO_3$, and 20 mM Hepes, pH 7.4) containing 3 mM glucose and 1.1 mg/mL of collagenase from *Clostridium histolyticum* (Type XI; Sigma, Cat. No. C7657) was used, and the islets isolated were cultured in Hepes-buffed RPMI-1640 (Wako) supplemented with 10% FBS (Hyclone), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Wako). To express Insulin-GLase, the islets were cultured for 2 days in 2 mL of the culture medium on glass-bottomed dishes (Mat-Tek, Cat. No. P35G-0-14-C, Ashland, MA) coated with Matrigel basement membrane matrix (Corning) and then infected by 6 μL of the adenoviral solution (4.8×10^6 pfu/dish) for 24–48 h.

2.4. Establishment of a clonal INS-1E cell line stably expressing Insulin-GLase (iGL cells)

The rat pancreatic beta cell line INS-1E was kindly provided by Dr. Wollheim [4] and cultured in HEPES-buffed RPMI-1640 (Wako, Cat. No. 189-02145) supplemented with 5% FBS (HyClone), 1 mM pyruvate, 50 μM 2-mercaptoethanol, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Wako). To establish a clonal INS-1E cell line stably expressing Insulin-GLase, INS-1E cells were transfected with pcDNA3-hINS-GLuc [3] using LipofectAMINE 2000 (Invitrogen), and selected with 200 μg/mL of G418 (Wako) in the culture medium. The cells transfected for 48 h were trypsinized, dispersed by repeated pipetting, diluted, and seeded in a 100-mm tissue culture dish (Falcon, Cat. No. 353003) with 1×10^6 cells per dish. The medium containing G418 was changed every 3 days and the cells were further incubated for 3 weeks. The colonies were formed as islet-like spheroids, and ~400 colonies were picked up and transferred into 96-well tissue culture plates (Falcon, Cat. No. 353072). After incubating for 3 days, the luminescence activity in the culture medium was measured using a Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) as follows: The culture medium (25 μL) was placed in a 96-well black plate (Berthold) and the luminescence activity was determined for 1 s by injection of 25 μL of PBS (D-PBS(-), Wako) containing 0.25 μg of coelenterazine (JNC Corporation, Tokyo, Japan). The spheroids with luminescence activity were selected, trypsinized and subcultured. The highest luminescent clone, in which secretion of Insulin-GLase was highly stimulated by high glucose (20 mM), was selected and named iGL. The secretion of Insulin-GLase into the culture medium was confirmed with Western blot analyses as described below.

2.5. Measurement of luminescence activity of Insulin-GLase in the culture medium and cell lysate of iGL cells stimulated by high glucose using a luminometer

iGL cells were seeded at $0.5–4 \times 10^5$ cells in 6-well plates coated with poly-D-lysine (Falcon, Cat. No. 356413), and cultured for 1–7 days (the medium was changed on the 3rd or 4th day). After washing twice with Krebs-Ringer Hepes buffer (KRH: 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, and 10 mM Hepes, pH 7.4) containing 0.01% BSA in the absence of glucose, the cells were pre-incubated for 1 h with the 0.01% BSA-KRH. After pre-incubation, the cells were washed twice with the 0.01% BSA-KRH and further incubated for 1 h with 2 mL of 0.01% BSA-KRH with or without 20 mM glucose. To determine secreted Insulin-GLase from cells, the culture medium was collected by centrifugation at 300 × g for 5 min at 4 °C to remove detached cells. To determine Insulin-GLase in cells, cells were lysed with 200 μL of Passive lysis buffer (Promega), and the cell lysate was obtained by centrifugation at 15,000 × g for 10 min at 4 °C to remove insoluble proteins. The culture medium (1 μL) or cell lysate (1 μL) was used for measuring the luminescence activity of GLase by the addition of 50 μL of PBS containing 0.25 μg of coelenterazine. The initial maximal light intensity was measured in 0.1 s intervals for 10 s using an Atto (Tokyo, Japan) AB2200 luminometer (ver. 2.61D) equipped with a R4220P photomultiplier (Hamamatsu Photonics, K.K.) in the presence of a neutral density filter. Luminescence activity per well was expressed as the mean values ± S.D. (n = 3).

2.6. Western blot analyses

Western blot analyses were performed using anti-GLase (Prolume, Pinetop, AZ) and anti-insulin (H-86, Santa Cruz Biotechnologies, Santa Cruz, CA) rabbit polyclonal antibodies as follows. INS-1E and iGL cells (1×10^6 cells) cultured for 3 days in a 60-mm dish (Falcon, Cat. No. 353002) were washed 3 times and incubated for 1 h with KRH (without glucose). After washing twice with KRH, the cells were further incubated for 1 h with 1 mL of KRH containing 20 mM glucose, and then the culture medium was collected in a microtube and centrifuged at 300 × g for 5 min at 4 °C. To analyze cell lysate, the cells were collected in KRH using a silicone blade cell scraper (Sumitomo Bakelite Co., Tokyo, Japan), and sonicated for 10 s on ice with an UP50H ultrasonic processor with 1 mm diameter tip (intensity = 0.4, Hielscher, Teltow, Germany). Protein concentration was determined using a