論 文 要 旨

Characterization of preptin-induced insulin secretion in pancreatic β-cells.

膵臓β細胞における誘導インスリン分泌の特性評価

鄭凱駿

【序論および目的】（適宜、項目をたてて、必ず２頁で記載する）

Preptin is a novel peptide that can be purified from the secretory granules of cultured murine b-cells. It is 34 amino acids long and corresponds to Asp69–Leu102 of proinsulinlike growth factor 2 (proIGF2E; Buchanan et al. 2001). This peptide belongs to the insulin, gastric intestinal peptide, and endocrine peptide family. Insulin secretion can be stimulated by both nutrient and non-nutrient secretagogues. The aim of this study is to characterize the effect of preptin on insulin secretion and to investigate the potential mechanism(s) responsible for this action of preptin.

【材料および方法】

Animals Wistar rats weighing 150–250 g were obtained from the Animal Center of Chi-Mei Medical Center. Rats were housed in a temperature-controlled room and kept on a 12 h light:12 h darkness cycle.

Total of 16 rats were segregated into two groups of eight animals each. After 12-h fasting, blood samples were withdrawn from tail vein and glucose in plasma was estimated for basal reading (0 min). Then, one group of the animals was treated with preptin (0.1 mg/kg) through i.v. injection into tail vein. Another group receiving same injection of vehicle at same volume was taken as control. Thirty minutes after the treatment, glucose solution at a dose of 1 g/kg body weight was administered orally. Blood samples were withdrawn at indicated time after the oral glucose load and the glucose in plasma was estimated. All experiments were carried out in animals under anesthesia with 2% isoflurane. Also, the insulin level in blood was determined using ELISA kit (Mercodia, Uppsala, Sweden). Cell line and culture conditions Mus musculus insulinoma cell line Min 6 cells (from Prof. Susumu Seino, Kobe University, Kobe, Japan) were culturedin a RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS (Biologic Industries, Kibbutz Beit Haemek, Israel), penicillin (100 IU/ml), streptomycin (100 mg/ml) (both from Sigma), and amphotericin B (2.5 mg/ml; Gibco).

The cells were subcultured once weekly by trypsinization (Gibco), and the medium was changed every 3–4 days. For the experiments, the cells were seeded on round (25 mm diameter) sterile glass coverslips and cultured for 48–72 h inRPMI 1640 medium supplemented as earlier. Measurement of insulin secretion To explore whether preptin has direct effects on insulin secretion, we performed in vitro secretion experiments using

Min 6 cells. Min 6 cells were seeded at 1x10 5 cells per well density in 1 ml DMEM in 12-well plates 24 h before glucosestimulated insulin secretion (GSIS). Briefly, on the day of the study, cells were pretreated with low glucose (2.0 mM) DMEM for 2 h and then replaced with medium consisting 1% BSA. Before GSIS, isolated islets were washed with low glucose (2.0 mM) medium. The cells for GSIS were treated with chelerythrine (PKC inhibitor) or U-73122 (PLC inhibitor) (both from RBI; Natick, MA, USA) at desired concentrations or vehicle at same volume as control for 30 min. Then, all cells were incubated with preptin at

desired concentrations for 1 h. Also, glibenclamide (Sigma) was treated with cells in another group at the same manner.

After the collection of media to store at at -20 ℃, insulin levels in the media were determined using insulin ELISA kit Measurement of intracellular calcium concentrations The changes in the intracellular calcium concentration were detected using the fluorescent probe fura-2 (Lee et al. 2007). Min 6 cells were placed in buffered physiological saline solution containing 140 mM NaCl, 5.9 mM KCl, 1.2 mM CaCl2, 1.4 mM MgCl2, 11 . 5 mM glucose, 1.8 mM Na_2HPO_4, and 10 mM Hepes–Tris, to which 5 mM fura-2 was added, and the cells were incubated for 1 h in humidified 5% CO_2 and 95% air at 37 8C. The cells were washed and incubated for an additional 30 min in PSS. The Min 6 cells were inserted into a thermostated (37 ℃) cuvette containing 2 ml calcium-free PSS and various doses of preptin or inhibitor as indicated. The fluorescence was continuously recorded using a fluorescence spectrofluorometer (Hitachi F-2000). Values of $[Ca^{2+}]_i$ were calculated from the ratio RZ F340/F380 by the formula $[Ca^{2+}]_i$ZKdB (RKRmin) /(RmaxKR), where Kd is 225 nM, F is the fluorescence, and B is the ratio of the fluorescence of the free dye to that of the Ca^{2+}-bound dye measured at 380 nm. R_{max} and R_{min} were determined in separate experiments using preptin to equilibrate $[Ca^{2+}]_i$ with ambient $[Ca^{2+}]$ (R_{max}) and adding 0.1 mM $MnCl_2$ and 1 mmol/l EGTA (R_{min}). Background autofluorescence was measured in unloaded cells and was subtracted from all measurements Western blotting analysis Specific protein expression levels in Min 6 cells were determined by western blotting analysis. Proteins extracted using radioimmunoprecipitation assay buffer were separated by SDS–PAGE, electrotransferred, and immobilized on a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBS-T) and incubated for 2 h. The membrane was then washed in PBS-Tand hybridized with IGF2 receptor (IGF2R) antibody (Santa Cruz), which were diluted to a suitable concentration in PBS-T for 16 h. Incubation with secondary antibodies and the detection of the antigen–antibody complex were performed using an ECL kit (Amersham Biosciences). Immunoblot densities were quantified using a laser densitometer.

【結　果】

We tested the role of intracellular calcium ions ($[Ca^{2+}]_i$) in insulin secretion caused by preptin. After incubation with preptin, the concentration of $[Ca^{2+}]_i$ was significantly raised in Min 6 cells. Preptin (from 10^{x-9} mol/l) produced a concentration-dependent increase in $[Ca^{2+}]_i$ concentration in Min 6 cells. Western blotting analysis of the membrane fraction prepared from Min 6 cells showed the expression of IGF2R. The presence of IGF2R in pancreatic b-cells can thus be identified. Effect of IGF2R antibodies on the action of preptin in Min 6 cells In the presence of antibodies specific to IGF2R, the effects of preptin were abolished. The increase in insulin secretion and the higher of calcium ion concentration caused by preptin were both markedly inhibited by the antibodies specific to IGF2R. This result implied the IGF2R as mediators of the actions of preptin.In preptin (10^{x-9} mol/l)-treated Min 6 cells, increase in insulin secretion was reduced by U73122 at a concentrationdependent manner from 10^{K7} to 10^{K5} mol/l. However, it was not modified by 10^{K5} mol/l of U73343, the inactive PLC inhibitor that is widely used as negative control.

【結論及び考察】

In conclusion, we suggest that preptin, an endocrine peptide secreted from pancreatic b-cells, can activate IGF2R linked to the PKC/PLC pathway to induce calciumdependent insulin secretion under high-glucose conditions. This finding provides new insight into the autocrine action of preptin. Also, activation of IGFRs by preptin is still not observed in other tissues and this shall be investigated in the future.

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論文審査の要旨

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Characterization of preptin-induced insulin secretion in pancreatic β-cells.

Preptin, which is known to be secreted from pancreatic β-cells, is a fragment of proIGF2 and the presence of IGF2 receptor (IGF2R) in pancreatic β-cells has been demonstrated. But the effect of preptin on insulin secretion is still unknown. At first, we found the increase of insulin secretion by preptin in fasting rats received glucose challenge test. Then, we investigated the potential mechanisms of the insulin secretion at single-cell level using Min 6 cell, the insulin-producing cell line. The following results were obtained:

1.Preptin enhances glucose-induced insulin release from Min6 cells to the level similar as glibenclamide which is known as the blocker of ATP-regulated KC channels for stimulation of insulin secretion.

2.Calcium ions (Ca^{2+}) are essential for insulin secretion and the regulation of intracellular Ca^{2+} $[Ca^{2+}]i$ mobilization by preptin was determined using fura-2 -loaded Min 6 cells. The data showed that preptin increases intracellular $[Ca^{2+}]i$ level in Min6 cells.

3.Western blotting analysis showed the expression of IGF2R in Min 6 cells. Using IGF2R antibodies instead of antagonist, the insulin secretion and calcium influx increased by preptin were blocked by IGF2R antibodies, indicating that the action of preptin is mainly mediated through IGF2 receptor.

4. The signal pathway of phospholipase C (PLC)/protein kinase C (PKC) is known to link with IGF2R. Then, the inhibitor for PLC or PKC was applied to complete the pharmacological characterization of this pathway in preptin-induced insulin secretion. The data showed that insulin secretion increased by preptin was reduced by the inhibitor specific to PLC or PKC. There indicate that the involvement of PLC/PKC signals is relevant to the potential mechanisms for increase in insulin secretion by preptin.

Taken together, it is indicated that preptin can interact with IGF2R to increase the PKC/PLC activity to induce calcium-dependent insulin secretion under high-glucose conditions. This finding provides new insight into the autocrine action of preptin. Then, the functional role of preptin to be contributed in scientific view of pancreatic β-cells is established. Therefore, this paper is proved to be valuable as a doctoral thesis.

最終試験の結果の要旨

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主査および副査の５名は、平成　25 年　2 月　25 日、学位申請者　鄭凱駿　君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

Question1). How about the preptin level in human? Dose preptin have any relationship with diabetes mellitus?

Reply: Preptin in human is known to be higher in female than in male. Also, there is still no report showing the relationship of preptin with diabetic disorders.

Question2). How about the IGF2 level in mouse and human blood?

Reply: It was about 15 ± 0.91 ng/mL in mouse as described previously. In human, it has been reported as 379 ng/mL to 387 ng/mL.

Question3). Why did you measure glucose and insulin level until 4 hours?

Reply: From the preliminary experiments, both levels of glucose and insulin reached plateau at 4 hours after glucose loading. Thus, I checked the changes of insulin and glucose levels until 4 hours to investigate the effects of preptin on glucose and insulin.

Question4). If you increase the dose of preptin, how dose it influence insulin level?

Reply: In cell level as shown in Figure 2, the action of preptin reaches the maximum because the effect of preptin at 10^{-6} M was the same as that at 10^{-7} M without statistical difference ($P>0.05$).

Question5). Why did you focus on intracellular Ca^{2+} in the preptin experiment? How about the basic level of the intracellular calcium concentration in the preptin experiment? Have you checked oscillation in the preptin experiment?

Reply: Because intracellular Ca^{2+} is essential for secretion of insulin, we checked the changes of intracellular Ca^{2+} by preptin. The basic level is about 200 nM that should

be added in Fig. 4. Also, the oscillation experiment has not been done in this study and will be included in our future research.

Question6). What kind of epitope of IGF2R, does the IGF2R antibody recognize? Is this antibody really specific for IGF2 receptor?

Reply: From the data of supplier, this rabbit polyclonal antibody recognizes amino acids 2192-2491 of human IGF-2R. This antibody is suggested to be specific for IGF2 receptor according to supplier.

Question7). Have you measured IGF2 receptor mRNA using RT-PCR? How about the IGF-2 receptor mRNA expression?

Reply: I did not measure the mRNA level of IGF2 receptor using RT-PCR in Min6 cell. However, this helpful comment is important and it would be conducted in our future research. Although we have done only the Western blotting analysis of IGF2 receptor, the protein expression is generally correlated with the gene expression. Thus, we suggest that changes in receptor protein could be related with that of receptor mRNA levels.

Question8). Have you checked the effect of preptin under the condition of calcium free and low glucose concentration in the preptin experiment? (Because the high glucose condition itself would increase calcium influx.)

Reply: I did not check the preptin effect under the calcium free and low glucose condition in this experiment. This is a good way to support the data for the major role of calcium ions in the action of preptin. I appreciate your helpful suggestion and will include this good idea into the protocol of our research in the future.

Question9). How do you think the future experiment in clinical research?

Reply: I think that preptin level in diabetic patients is still unclear and it must be characterized as soon as possible. Also, the role of preptin in pancreatic cancer needs to be identified because the IGF-2 receptor promotes the development of fetal pancreatic beta cells. Moreover, it should be identified that IGF2 may affect bone growth and effect of preptin on bone marrow and/or stem cells, would be helpful in clinical research.

Question10). What do you want to do in basic experiments of preptin in your future research?

Reply: It has been established that GLP-1 induces insulin secretion and protection against the damage of pancreatic beta-cell. Thus, I would like to know whether or not preptin shows the similar action. Then, the effect of preptin on the streptozotocin-induced damage of pancreatic beta-cells will be investigated in the future.

以上の結果から、５名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。