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

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Abstract

We aimed to characterize the effects of preptin on insulin secretion at the single-cell level, as well as the mechanisms underlying these changes, with respect to regulation by intracellular Ca^{2+} [Ca^{2+}]_i mobilization. This study assessed the effect of preptin on insulin secretion and investigated the link between preptin and the phospholipase C (PLC)/protein kinase C (PKC) pathway at the cellular level using fura-2 pentakis(acetoxymethyl) ester-loaded insulin-producing cells (Min 6 cells). Our results demonstrate that preptin promotes insulin secretion in a concentration-dependent manner. Using a PLC inhibitor (chelerythrine) or a PKC inhibitor (U73122) resulted in a concentration-dependent decrease in insulin

secretion. Also, preptin mixed with IGF2 receptor (IGF2R) antibodies suppressed insulin secretion in a dose-dependent manner, which indicates that activation of IGF2R is mediated probably because preptin is a type of proIGF2. In addition, preptin stimulated insulin secretion to a similar level as did glibenclamide. The activation of PKC/PLC by preptin stimulation is highly relevant to the potential mechanisms for increase in insulin secretion. Our results provide new insight into the insulin secretion of preptin, a secreted proIGF2-derived peptide that can induce greater efficacy of signal transduction resulting from PLC and PKC activation through the IGF2R.

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Introduction

Preptin is a novel peptide that can be purified from the secretory granules of cultured murine β -cells. It is 34 amino acids long and corresponds to Asp69–Leu102 of proinsulin-like growth factor 2 (proIGF2E; Buchanan *et al.* 2001). This peptide belongs to the insulin, gastric intestinal peptide, and endocrine peptide family. Several studies have shown that preptin can enhance insulin secretion, while infusion of isolated pancreases with preptin antibodies significantly reduces glucose-mediated insulin secretion (Yang *et al.* 2009).

Insulin secretion can be stimulated by both nutrient and non-nutrient secretagogues. Non-nutrient insulin secretagogues may modulate β -cell ion channels through the cAMP or phospholipase C (PLC) pathways (Harndahl *et al.* 2002), or they may increase the release of arachidonic acid (Konrad *et al.* 1994), thereby sensitizing these channels to primary stimuli. Neurotransmitters and hormones, such as acetylcholine, arginine vasopressin, or bombesin, that activate receptors coupled to the Ca^{2+} –phosphoinositide (PI) pathway cause a rise in the concentration of cytosolic-free Ca^{2+} ([Ca^{2+}]_i) and stimulate insulin secretion from normal and transformed β -cells in the presence of glucose (Garcia

et al. 1988, Gao *et al.* 1990, Regazzi *et al.* 1990, Schoff *et al.* 1995). Several lines of evidence, mainly from non-excitabile cells, suggest that protein kinase C (PKC), which is coactivated upon stimulation of receptors coupled to the Ca^{2+} –PI pathway, may regulate PLC-linked Ca^{2+} oscillations via a negative feedback loop (Woods *et al.* 1987, Cuthbertson & Chay 1991, Bird *et al.* 1993, Kawabata *et al.* 1996, Thomas *et al.* 1996, Codazzi *et al.* 2001, Young *et al.* 2002).

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.

Materials and Methods

Peptide synthesis and preparation of drugs

Mouse preptin (DVSTSQAVLPDDFPRYPVGKFFQYDT-WRQSAGRL) was synthesized by a solid-phase methodology with a fluorenylmethoxycarbonyl strategy using an automated peptide synthesizer (Model Pioneer; Applied Biosystems) and the crude peptide was purified by a reverse-phase HPLC (Delta 600 HPLC System; Waters, MA, USA) on a column of Develosil ODS-HG-5

(2×25 cm; Nomura Chemical, Aichi, Japan). The homogeneity of the purified peptide was confirmed by analytical HPLC, MALDI-TOF MS, and sequence analysis.

Determination of postprandial blood glucose in rats

Animals Male 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 ($25 \pm 1^\circ\text{C}$) and kept on a 12 h light:12 h darkness cycle (light on at 0600 h). Water and standard laboratory diet were freely available throughout. Studies were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal experiments were approved by the Regional Ethics Committee for Animal Research in Chi-Mei Medical Center (Tainan, Taiwan).

Effect of preptin on postprandial blood glucose was carried out mainly according to a previous report (Baron 1998). A 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 animals was treated with preptin (0.1 mg/kg) through i.v. injection into the tail vein. Another group receiving the same injection of vehicle at the 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 the 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. 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 cultured in 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 in RPMI 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 1×10^5 cells per well density in 1 ml DMEM in 12-well plates 24 h before glucose-stimulated 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 in the same manner. After the collection of media to store at -20°C , insulin levels in the media were determined using an insulin ELISA kit (Mercodia).

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 CaCl_2 , 1.4 mM MgCl_2 , 11.5 mM glucose,

Figure 1 Effects of preptin on changes in blood glucose and insulin levels in fasted Wistar rats after glucose (1 g/kg) loading. (A) Changes of blood glucose and (B) changes of blood insulin. There was a decrease in blood glucose and increase in blood insulin by i.v. injection of preptin (0.1 mg/kg) in fasting rats. Each point represents mean $\pm$ S.E.M. from eight rats. * $P < 0.05$ vs compared with control group.

1.8 mM Na_2HPO_4 , and 10 mM Hepes–Tris, to which 5 μM fura-2 was added, and the cells were incubated for 1 h in humidified 5% CO_2 and 95% air at 37 °C. The cells were washed and incubated for an additional 30 min in physiological saline solution (PSS). The Min 6 cells were inserted into a thermostated (37 °C) 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 $[\text{Ca}^{2+}]_i$ were calculated from the ratio $R = F340/F380$ by the formula $[\text{Ca}^{2+}]_i = K_d B (R - R_{\min}) / (R_{\max} - R)$, where K_d 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 $[\text{Ca}^{2+}]_i$ with ambient $[\text{Ca}^{2+}]$ ($R_{\max}$) 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–T and 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.

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. for the number (n) of samples in one group as indicated. Statistical analysis was carried out using one-way ANOVA analysis and Newman–Keuls *post-hoc* analysis. A P value of 0.05 or less was considered significant.

Results

Effect of preptin on blood glucose and insulin levels in rats challenged with glucose

In fasted Wistar rats, preptin failed to affect the blood glucose level in preliminary experiments. Thus, we gave preptin to fasted Wistar rats receiving a 1 g/kg glucose challenge to investigate the effects of preptin. Preptin i.v. injected into rats resulted in a frank decrease in blood glucose

Figure 2 Preptin increased insulin secretion in Min 6 cells. In the presence of 25 mM glucose, insulin secretion was significantly potentiated by preptin from 10^{-9} to 10^{-6} mol/l, but it was not changed in medium containing low glucose (5 mM). Results expressed as mean $\pm$ S.E.M. are obtained from six independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared with control group.

and a marked increase in blood insulin as compared with the vehicle-treated group (Fig. 1).

Effect of preptin on insulin secretion in Min 6 cells

To determine the effect of preptin on insulin secretion, we used Min 6 cells to investigate insulin secretion under high-glucose conditions (25 mmol/l) because preptin failed to modify insulin secretion in this cell line in normal medium. The glucose-induced insulin release was potentiated by preptin in a concentration-dependent manner: preptin at a concentration of 10^{-9} , 10^{-8} , 10^{-7} , and 10^{-6} mol/l increased insulin release significantly ($P < 0.05$), while preptin at a concentration higher than 10^{-6} mol/l showed same action as that at 10^{-6} mol/l (Fig. 2). These results indicated that preptin enhances glucose-induced insulin release from pancreatic β -cells in a concentration-dependent manner.

Figure 3 Under high-glucose conditions (25 mmol/l), insulin secretion was markedly raised in Min 6 cells treated with glibenclamide (10^{-6} mol/l) compared with that induced by preptin (10^{-6} mol/l). *** $P < 0.005$ compared with control group.

Comparison of the effects of glibenclamide and preptin on insulin secretion

In general, glibenclamide is widely used to increase insulin secretion. Glibenclamide increased insulin secretion from Min 6 cells significantly at a concentration of 10^{-6} mol/l. Preptin at a concentration of 10^{-6} mol/l increased insulin secretion to a level similar to that induced by glibenclamide at 10^{-6} mol/l (Fig. 3).

Changes in $[Ca^{2+}]_i$ caused by preptin in Min 6 cells

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^{-9} mol/l) produced a concentration-dependent increase in $[Ca^{2+}]_i$ concentration in Min 6 cells (Fig. 4).

Identification of the presence of IGF2R in Min 6 cells

Western blotting analysis of the membrane fraction prepared from Min 6 cells showed the expression of IGF2R (Fig. 5). The presence of IGF2R in pancreatic β -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 (Fig. 6). The increase in insulin secretion and the higher calcium ion concentration caused by preptin were both markedly inhibited by the antibodies specific to IGF2R. This result implied that IGF2R is a mediator of the actions of preptin.

Figure 4 Increase in calcium influx by preptin in Min 6 cell under high-glucose conditions (25 mmol/l). The $[Ca^{2+}]_i$ levels determined by fura-2 were concentration dependently increased by preptin. Results expressed as mean $\pm$ s.e.m. were obtained from six independent experiments.

IGF2R

Min 6 5 μ l 10 μ l 15 μ l 20 μ l

Figure 5 Western blotting analysis of the membrane fraction from Min 6 cells shows the expression of IGF2R. Min 6 cells were fractionated and membrane fractions were probed with IGF2R antibodies as described in the Materials and Methods section. Expression of IGF2R was raised with the increase in loading membrane protein from lane 1 to 4 running duplicated. Immunoblot analyses were repeated three times, showing similar results.

Effect of U73122, a PLC inhibitor, on the action of preptin in Min 6 cells

In preptin (10^{-9} mol/l)-treated Min 6 cells, increase in insulin secretion was reduced by U73122 in a concentration-dependent manner from 10^{-7} to 10^{-5} mol/l (Fig. 7). However, it was not modified by 10^{-5} mol/l of U73343, the inactive PLC inhibitor that is widely used as negative control.

Effect of chelerythrine, a PKC inhibitor, on the action of preptin in Min 6 cells

In Min 6 cells treated with preptin (10^{-9} mol/l), as shown in Fig. 8, the concentration-dependent blockade of preptin-induced insulin secretion was also observed by chelerythrine from 10^{-7} to 10^{-5} mol/l.

Discussion

This study shows that i.v. injection of preptin at a dose of 0.1 mg/kg triggers a decrease in blood glucose level in fasted rats challenged by glucose. However, similar injection of preptin failed to modify blood sugar levels in normal rats. Otherwise, blood insulin level was raised by similar injection of preptin in fasted rats challenged by glucose. Thus, an acute injection of preptin induces a decline in plasma level of glucose connected with insulin secretion during glucose challenge in rats.

Min 6 cells are widely used in the study of insulin secretion (Nakashima *et al.* 2012). Therefore, we studied this cell line instead of primary cultured pancreatic cells obtained from killed animals for understanding the effect of preptin on insulin secretion (Fig. 9). Actually, preptin caused a concentration-dependent increase in insulin secretion from Min 6 cells. This action of preptin is only induced in

Figure 6 Effect of antibodies specific to IGF2R on the insulin secretion induced by preptin in Min 6 cells. (A) Changes in insulin secretion and (B) changes in calcium influx into Min 6 cells. Increase in insulin secretion by preptin (10^{-9} mol/l) was inhibited by antibodies specific to IGF2R in a concentration-dependent manner. Data represent mean $\pm$ S.E.M. from six experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared with each control.

high-glucose conditions (25 mmol/l) but not in normal medium. There are many factors that affect the β -cell glucose sensitivity. One of the possible mechanism(s) for this is that preptin stimulating insulin secretion acts not only at the level of the amplifying pathway but also the triggering pathway; otherwise, the final effect would be that of an imbalance between these pathways. Moreover, under low concentrations of glucose, β -cells are not saturating for the glucose transporter and/or the expressed hexokinase, resulting in a reduction of glucose metabolism. High-glucose concentrations stimulate insulin release and increase glucose sensitivity in terms of insulin secretion, electrical activity, and Ca^{2+} signaling for changes in glucose metabolism (Rajan *et al.* 1990, Martín *et al.* 1999). Also, as shown in Fig. 4, this action of preptin is produced in a calcium-dependent manner. Otherwise, it has been demonstrated that glucose-induced insulin secretion is dependent on ATP-regulated K^+ channels (Rorsman & Trube 1985).

Then, we used Min 6 cells to compare the insulin secretion action of preptin with glibenclamide, which is widely used to stimulate insulin secretion (Tominaga *et al.* 1995). The data

indicate that Min 6 cells exposed to 10^{-6} mol/l preptin can produce an effect on insulin secretion similar to that of glibenclamide at 10^{-6} mol/l. There was no significant difference between these two stimuli in insulin secretion. It has been established that glibenclamide acts as a blocker of ATP-regulated K^+ channels in pancreatic β -cells, thereby causing Ca^{2+} influx and a subsequent increase in $[\text{Ca}^{2+}]_i$. Glibenclamide can cause the dose-dependent inhibition of the K_{ATP} channels to increase insulin secretion from β -cells (Tominaga *et al.* 1995). We found that an increase in insulin secretion by preptin was produced at a concentration similar to glibenclamide. The effective concentration of preptin seems higher as a peptide. It is possible that IGF2Rs are changed in cultured cells. Thus, preptin needs higher amounts to activate the IGF2Rs. Nevertheless, this is the first report that compared the efficiency of glibenclamide with preptin for insulin secretion *in vitro*.

As preptin is a fragment of a larger precursor (proIGF2), it is possible that preptin acts on cells through the IGF2R. IGF2R is a type-I transmembrane glycoprotein containing a large N-terminal extracellular region, a single membrane-spanning region, and a small cytoplasmic tail (Takasu *et al.* 1989, Ghosh *et al.* 2003). Actually, we are the first to identify the presence of IGF2R in Min 6 cells (Fig. 5). So far as we know, there is no direct antagonist of IGF2R. Thus, we used the IGF2R antibody to determine whether preptin acts via IGF2R. In this study, we found that insulin secretion of preptin was reduced when the IGF2R was blocked by antibodies, suggesting that the insulin secretion of preptin is mediated by the IGF2R pathway. However, it is possible that the IGF2R antibody bound to preptin directly. Thus, we investigated the changes of signals related to IGF2R in advance.

The signal transduction mechanisms involved IGF1- and IGF2-mediated induction of mobilization of Ca^{2+} from the endoplasmic reticulum with an activation of PLC. This effect

Figure 7 Effect of PLC inhibitor (U73122) on the insulin secretion induced by preptin in Min 6 cells. Action of preptin was reduced by U73122 in a concentration-related manner. Data represent mean $\pm$ S.E.M. from six experiments. * $P < 0.05$, ** $P < 0.01$ compared with each control.

Figure 8 Effect of the PKC inhibitor chelerythrine on insulin secretion induced by preptin in Min 6 cells. Action of preptin was inhibited by chelerythrine in a concentration-related manner. Data represent mean $\pm$ S.E.M. from six experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared with each control.

of IGF1 or IGF2 on Ca^{2+} mobilization indicates that IGF1 or IGF2 increases inositol 1,4,5-triphosphate formation in different cell types (Rogers & Hammerman 1988, Guse *et al.* 1992, Muto *et al.* 1997). In Min 6 cells and primary β -cells, PLC-linked agonists at low near physiological concentrations cause Ca^{2+} oscillations whose frequency is determined by the extracellular agonist concentration, whereas the amplitude remains constant (Gao *et al.* 1990, Cuthbertson & Chay 1991, Konrad *et al.* 1994, Thomas *et al.* 1996, Zawulich & Zawulich 1996). This result indicates that the cytosolic Ca^{2+} signal evoked by PLC-linked agonists might be primarily frequency encoded. Thus, we used a PLC inhibitor (U73122) to determine whether PLC is the pathway affected by preptin (Thomas *et al.* 1996). In this study, the stimulatory effect of preptin on insulin secretion was almost

completely abrogated in Min 6 cells preincubated with U73122 at concentrations from 10^{-7} to 10^{-5} mol/l. The results indicate that the PLC pathway is involved in the actions of preptin after activation of IGF2R.

PKC is known to be activated and translocated to the plasma membrane during calcium signaling. The involvement of PKC has been previously implied in the regulation of PLC-generated $[\text{Ca}^{2+}]_i$ oscillations in several non-excitable cell types (Thomas *et al.* 1996, Nakashima *et al.* 2012). To understand the effects of PKC-mediated modulation of preptin-induced $[\text{Ca}^{2+}]_i$ oscillations and of PLC-induced $[\text{Ca}^{2+}]_i$ oscillations in insulin-producing cells, it is desirable to inhibit or inactivate PKC directly. The role of PKC was thus investigated using chelerythrine that can inhibit PKC in a competitive manner with respect to phosphate acceptor (Herbert *et al.* 1990). The effect of preptin enhancement on insulin secretion was sensitive to the PKC inhibitor. In the presence of chelerythrine, the action of preptin to stimulate insulin secretion of Min 6 cells was attenuated in a concentration-dependent manner. These results demonstrate that the activation of PKC could be a potential mechanism mediating the stimulation of insulin secretion from β -cells in response to preptin. Taken together, the involvement of the PLC–PKC pathway in the regulation of insulin after stimulating IGF2R by preptin is evident in Min 6 cells (Fig. 9).

In conclusion, we suggest that preptin, an endocrine peptide secreted from pancreatic β -cells, can activate IGF2R linked to the PKC/PLC pathway to induce calcium-dependent insulin secretion under high-glucose conditions. This finding provides new insight into the autocrine action of preptin. Also, activation of IGF1R by preptin is still not observed in other tissues and this shall be investigated in the future.

Figure 9 Hypothetical model for the regulation of insulin secretion by preptin and IGF2. Preptin influences the secretory process through the activation of IGF2 as a consequence of an influx of extracellular Ca^{2+} . Receptor-operated non-nutrients that are coupled to PLC may activate Ca^{2+} -dependent protein kinases by the IP3-induced release of Ca^{2+} from intracellular stores and activate conventional and/or novel isoforms of PKC by the generation of DAG.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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