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Epidermal growth factor increases insulin secretion and lowers blood glucose in diabetic mice

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Abstract

Epidermal growth factor (EGF) is synthesized in the pancreas and diabetic animals have low levels of EGF. However, the role of EGF in regulating the major function of the pancreas, insulin secretion, has not been studied. Here, we show that EGF rapidly increased insulin secretion in mouse pancreatic islets, as well as in a pancreatic β-cell line. These events were dependent on a Ca^{2+} influx and phospholipase D (PLD) activity, particularly PLD2, as determined using pharmacological blockers and molecular manipulations such as over-expression and siRNA of PLD isozymes. In addition, EGF also increased plasma insulin levels and mediated glucose lowering in normal and diabetic mice. Here, for the first time, we provide evidence that EGF is a novel secretagogue that regulates plasma glucose levels and a candidate for the development of therapeutics for diabetes.

Keywords: epidermal growth factor • insulin secretion • phospholipase D2 • glucose homeostasis

Introduction

The main function of pancreatic β cells is to synthesize and secrete insulin at appropriate rates to limit blood glucose fluctuations. Excessive secretion of insulin causes hypoglycemia, and insufficient secretion leads to diabetes [1]. Therefore, it is not surprising that insulin secretion is subject to very tight control to ensure glucose homeostasis in the body. Insulin is stored in secretory granules in pancreatic β cells and, upon stimulation with secretagogues, is released by exocytosis [2]. The level of β-cell activity is determined by several different stimulators, including glucose, amino acids, fatty acids, neurotransmitters and hormones. In spite of intensive studies, the processes that are involved in this stimulus–secretion coupling and that maintain exquisite control of insulin release are still incompletely understood [1, 3–5].

Epidermal growth factor (EGF) is an important growth factor for the proliferation of different types of cells, especially fibroblasts and epithelial cells [8]. EGF can also induce secretion events, including acrosomal exocytosis and the secretion of several hormones [9, 10]. Some members of the EGF family are proposed to have a role in the development of the pancreas. EGF and leukemia inhibitory factor (LIF) treatment *in vitro* generated an insulin-producing β-cell mass [11]. The epidermal growth factor receptor (EGFR) is expressed throughout the human foetal pancreas, and mice lacking EGFR show abnormal pancreatic islets [12]. EGF is also related to the insulin content and regeneration of rat pancreatic β cells. [13–15]. EGF is also produced in the pancreas, and its circulating levels, along with EGFR, are reduced in diabetic animals [6, 7, 16]. However, the

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role of EGF in glucose regulation through the modulation of pancreatic functions, such as insulin secretion, has not been studied yet.

Insulin secretion is mainly triggered by the elevation of intracellular Ca^{2+}, but it can be modulated by several cellular signals such as protein kinases and phospholipases. Of these signals, mammalian phospholipase D (PLD) is a membrane-bound enzyme that hydrolyzes phosphatidyl choline (PC) to generate a multifunctional lipid, phosphatidic acid (PA), in response to a variety of signals, including growth factors [17]. PA is an intracellular lipid second messenger that is involved in multiple physiological events. These findings suggest that agonist-induced PLD activation may play roles in multiple signalling events [18, 19]. To date, two types of mammalian PLD, PLD1 and PLD2, have been cloned. They share a sequence homology of around 50% and contain similar regulatory domains. However, they show differences in localization and regulatory protein interactions [20]. PLD activity may be involved in various trafficking processes, particularly in the regulation of exocytosis [18]. PLD1 and PLD2 regulate different phases of exocytosis in mast cells by a two-step process [21]. In addition, PA is an important mediator of insulin exocytosis [22]. However, the specific regulation of PLDs by secretagogues remains unclear. Interestingly, PLD activity is inhibited in diabetic animals [23]. Therefore, it is important to study how secretagogues or receptor-mediated PLD activation is involved in insulin secretion.

Here, we show that a brief exposure to EGF stimulates insulin secretion in a glucose-independent manner via a Ca^{2+} influx- and PLD2-dependent mechanism. Furthermore, we show that EGF is a novel secretagogue that lowers plasma glucose levels in normal and diabetic mice, suggesting the potential for EGF treatment in diabetes.

Materials and methods

Materials

The enhanced chemiluminescence (ECL) kit was purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom); [^{3}H]myristic acid from Dupont NEN (Boston, MA, USA); Silica Gel 60 thin-layer chromatography plates from MERCK (Darmstadt, Germany); Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and LipofectAMINE from Invitrogen (Carlsbad, CA, USA); Fetal calf serum from HyClone (Logan, UT, USA); EGF from the Daewoong Pharmaceutical Company (Seoul, Republic of Korea); AG1478 from CalBiochem (San Diego, CA, USA); Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgA, IgG and IgM from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD, USA); and Fluo-3 AM from Molecular Probes (Eugene, OR, USA). A polyclonal antibody (mSTP4) recognizing both PLD1 and PLD2 was produced as described previously [24]. A PLD2-specific antibody was generated as described previously [25]. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

PLD constructs

The full-length cDNAs of rat PLD1 or human PLD2 were ligated into a pcDNA 3.1 vector for transfection into cells.

siRNA sequences

The siRNA of 21-mers corresponding to mouse PLD1 (nucleotides 1099–1119, AACACGUUAGCUAAGUGGUAU) or PLD2 sequences (nucleotides 2539–2559, AACUCCAUCCAGGCUAUUCUG) were purchased from Dharmacon Research Inc. (Lafayette, CO, USA). Results of a BLAST search of all siRNA sequences revealed no significant homology to any other sequences in the database.

$[Ca^{2+}]_i$ measurement

Changes in intracellular Ca^{2+} levels were monitored using a Ca^{2+}-sensitive dye under a confocal microscope. Cells were loaded with 2 μl Fluo-3 AM for 40 min. at room temperature. After washing with Krebs-Ringer bicarbonate (KRB; 129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.7 mM glucose and 10 mM HEPES, pH 7.4) buffer, the cells were further incubated for 15 min. in the absence of Fluo-3 AM to de-esterify the dye. To exclude the possible effects of dye loading, we normalized levels with saponin at the end of the experiments. To normalize the data, we measured the residual fluorescence (Fo) at the end of the experiment, and subtracted that from the fluorescence under experimental conditions (F). Excitation of Fluo-3 AM was performed at 488 nm by an argon laser, and the emission range was 515 nm. Images were captured on an inverted confocal microscope (Zeiss LSM 510 Meta, Oberkochen, Germany) with a 20x objective lens.

PLD activity assay in cells

Cells grown in 6-well plates were washed twice with KRB, and were then labelled with [^{3}H]myristic acid for 4 hrs at 37°C in the KRB solution. PLD activity was assayed by measuring the formation of phosphatidylbutanol (PBt) [26]. The intensities of PBt spots in the presence of 0.4% 1-butanol were measured, and PLD activity was obtained by subtracting the corresponding intensities of spots obtained in the absence of 1-butanol.

Immunoblot analysis

Proteins were denatured by boiling for 5 min. at 95°C in Laemmli sample buffer [27], separated by SDS-PAGE, and immunoblotted, as described previously [26].

Cell culture

The mouse insulin-producing MIN6m9 cells provided by Dr. Susumu Seino (Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan) were used between passages 19 and 25,

and were cultured in DMEM containing 25 mM glucose, 10 mM HEPES, 10% (v/v) foetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified CO_2-controlled (5%) incubator. MIN6 cells were transfected using LipofectAMINE, as described previously [26] Transfection efficiency is about 30–40% when using LipofectAMINE.

Preparation of pancreatic islets

Pancreatic islets were isolated from 7- to 8-week-old male BALB/c mice (Hyochang Science, Republic of Korea), as described previously [28]. Isolated islets were transferred to a 12-well plate, with 10–15 islets per well. We used the same number of islets in a same set of experiment. The islets were maintained for up to 2 days in RPMI1640 medium containing 5 mM glucose and 10% foetal calf serum, and supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin.

Insulin secretion assay

Batches of 10–15 isolated islets or 1 x 10^6 cells/well grown in 12- or 24-well plates were washed twice with KRB supplemented with 0.2% bovine serum albumin (BSA), and were then incubated for 60 min. at 37°C in the KRB solution. We used the same number of islets in a same set of experiment. At the end of incubation, the solutions were replaced with fresh KRB containing test reagents and incubated for the designated time. The incubation medium was sampled and centrifuged to remove cells, and the supernatant was assayed for insulin levels with a rat insulin radioimmunoassay (RIA) kit (Linco, St. Louis, MO, USA, Cat. # RI-13K).

Measurement of plasma glucose and plasma insulin levels

Seven- to 8-week-old male ICR mice were purchased from Hyochang Science (Seoul, Republic of Korea). C57BLKSJ-*db/db* mice were purchased from SLC (Japan). After fasting for 6 hrs, ICR or C57BLKSJ-*db/db* mice were intravenously injected with saline, EGF, insulin or glucose in the tail vein and blood samples (0.1 ml) were subsequently collected. Concentrations of plasma glucose were measured by the glucose oxidase method with a portable glucose meter (Gluco-Dr, Republic of Korea). Plasma was separated by centrifugation, and the plasma insulin assay was performed using a RIA kit. Animal care was conducted in accordance with the guidelines of our institution.

Measurement of plasma EGF levels

Seven- to 8-week-old male ICR mice were purchased from Hyochang Science (Seoul, Republic of Korea). After fasting for 6 hrs, ICR mice were orally injected with saline or glucose, and blood samples (0.1 ml) were collected in EGTA coated tubes. Concentrations of plasma EGF levels were measured by EGF ELISA kit (KOMA Biotech, Republic of Korea). Animal care was conducted in accordance with the guidelines of our institution.

Statistical analysis

Results are presented as mean ± SE or mean ± SD. The statistical significance of differences between means was assessed by Student's *t*-test. $P < 0.05$ was regarded as statistically significant.

Results

EGF stimulates insulin secretion in MIN6 cells

EGF is produced in the pancreas and has pancreatic effects, and its circulating levels are altered in diabetes [6, 7]. To determine whether EGF could stimulate insulin secretion, we treated mouse MIN6 insulinoma cells with EGF. EGF showed time- and dose-dependent stimulation of insulin secretion from MIN6 cells, with kinetics more rapid than glucose (Fig. 1A and B). EGFR is expressed throughout the human foetal pancreas [12]. Moreover, the effect of EGF on the release of insulin is quite rapid, and clearly detectable after 1 min. Therefore, it is important to determine if EGF regulates insulin secretion by acting directly through its own receptor. To address this question, the effect of blocking of EGFR on the EGF-induced secretion of insulin was examined. The EGFR blocker (AG1478) completely inhibited the EGF-induced insulin secretion (Fig. 1C). Therefore, EGF induces insulin secretion through its own receptor. In order to exclude the possible cross-talk between EGF and glucagon-like peptide-1 (Glp-1) signalling, the effect of blocking of the Glp-1 receptor on the EGF-induced secretion of insulin was examined. The Glp-1 receptor blocker (EXENDIN fragment 9–39) had no effect on the EGF-induced insulin secretion in MIN6 cells (Supplement Fig. 1). Therefore, EGF induces insulin secretion through its own receptor and not through Glp-1 cross-talk. To determine whether insulin secretion by EGF was additive by glucose treatment, we tested the effect of a high level (11 mM) of glucose on EGF-induced insulin secretion. EGF increased insulin levels at a basal concentration of glucose (2.7 mM), and also additively increased glucose-induced insulin release at high (11 mM) glucose levels (Fig. 1D). Taken together, these data suggest that EGF, like glucose, is an initiator of insulin secretion in pancreatic β cells, and also indicate that the effect of EGF on insulin secretion is glucose-independent.

EGF-induced insulin secretion is dependent on Ca21 influx in MIN6 cells

Insulin secretion requires increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [2]. Figure 2A demonstrated that EGF stimulated an extra-cellular Ca^{2+} influx, which could be reduced by EGTA treatment. To determine the effect of Ca^{2+} influx on EGF-induced insulin secretion, we treated cells with either EGTA to block extra-cellular Ca^{2+} influx or 1,2-Bis(2-aminophenoxy) ethane

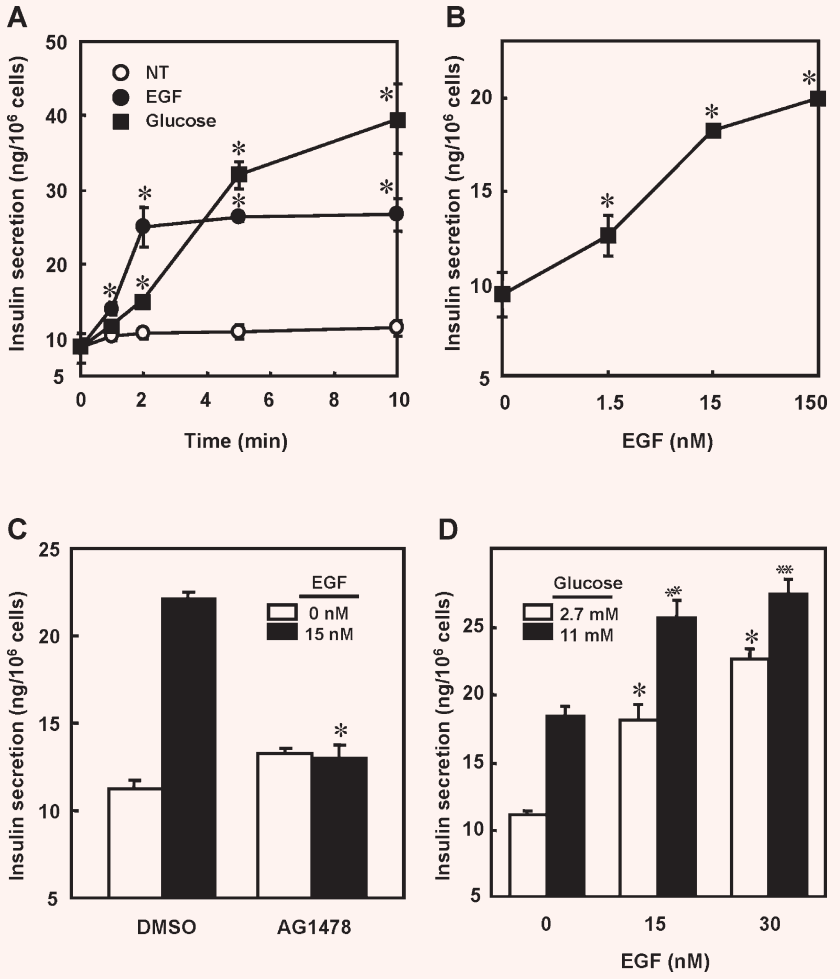

Fig. 1 EGF rapidly and glucose-independently stimulates insulin secretion in MIN6 cells. (**A–D**) The MIN6 cells were plated onto 24-well plates and grown for 24 hrs. The cells were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. (**A**) At the end of incubation, the solutions were replaced with fresh KRB containing none (NT), 15 nM EGF or 11 mM glucose, and were incubated for 0, 1, 2, 5 or 10 min. The incubation medium was sampled for measuring insulin levels. $^{*}P < 0.05$ compared with not-treated (NT) cells. (**B**) At the end of incubation, the solutions were replaced with fresh KRB containing 0, 1.5, 15 or 150 nM of EGF, and were incubated for 1 min. The incubation medium was sampled for measuring insulin levels. $^{*}P < 0.05$ compared with not-treated cells. (**C**) At the end of incubation, the solutions were replaced with fresh KRB containing none (untreated) or 10 μM AG1478 and were incubated for 30 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. $^{*}P < 0.05$ compared with EGF-treated cells. (**D**) At the end of incubation, the solutions were replaced with fresh KRB containing 0, 15 or 30 nM of EGF in the presence of 2.7 or 11 mM glucose, and were incubated for 5 min. The incubation medium was sampled for measuring insulin levels. * or $^{**}P < 0.05$ compared with 2.7 or 11 mM glucose-treated cells. (**A–D**) The data shown are the mean ± S.D. from two independent assays performed in duplicate.

Supplement Fig. 1 EGF-induced insulin secretion is not affected by Glp-1 receptor inhibitor. The MIN6 cells were plated onto 24-well plates and grown for 24 hrs. The cells were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. At the end of incubation, the solutions were replaced with fresh KRB containing none (untreated) or 100 nM EXENDIN fragment [9–39] and were incubated for 30 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. The data shown are the mean ± S.D. from two independent assays performed in duplicate.

N,N,N′,N′-tetra-acetic acid tetrakis(acet-oxymethyl ester) (BAPTA/AM) to block both extra-cellular Ca^{2+} influx and intracellular Ca^{2+} release. EGF-induced insulin secretion from MIN6 cells was reduced by Ca^{2+} chelators (Fig. 2B). The same results were also observed in RINm5F cells (data not shown). It would also be worthwhile to determine if the EGF effect acts on the L-type Ca^{2+} channels. This was confirmed by examining the effect of nifedipine, L-type Ca^{2+} channel inhibitor, rather than a non-selective chelator in the same experiment. The results showed that nifedipine blocked the EGF-dependent insulin secretion in a similar manner to that observed with the EGTA treatment (Fig. 2C). Taken together, these results suggest that the Ca^{2+} influx is necessary for EGF-induced insulin secretion, particularly through L-type Ca^{2+} channels.

PLD2 mediates EGF-dependent insulin secretion in MIN6 cells

Previous reports have suggested that PLD is an important molecule that mediates various exocytosis [18, 21, 22]. Furthermore, EGF is a strong agonist of PLD in various cells [29–31]. To investigate this, we first tested the PLD activity in MIN6 cells. PLD was rapidly activated (within 2 min.) by EGF stimulation (Fig. 3A). EGF-dependent insulin secretion was inhibited by treatment with 1-butanol, a PLD inhibitor, but not by *t*-butanol treatment as a control (Fig. 3B). These results suggest that PLD activity is necessary for EGF-induced insulin secretion. The same results were also observed in RINm5F cells (data not shown).

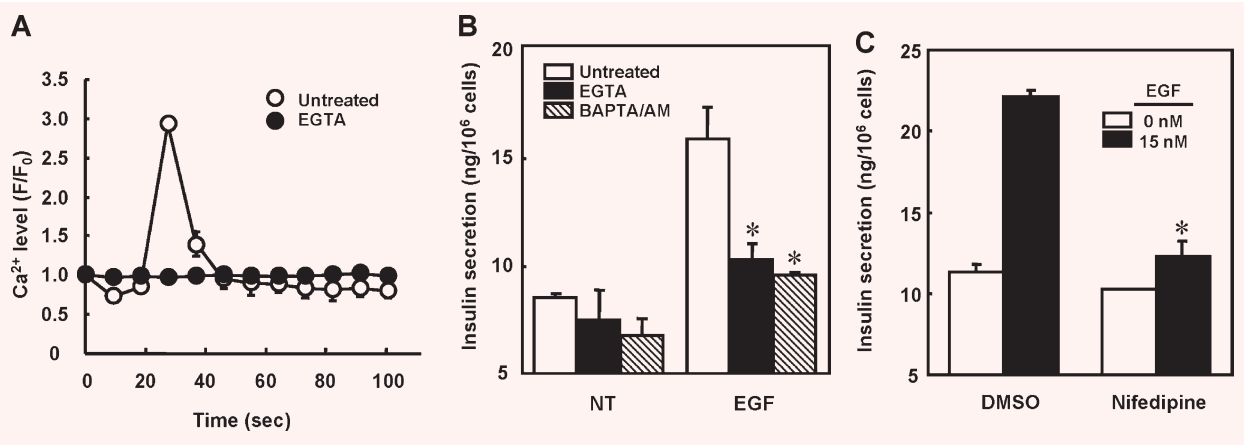

Fig. 2 Ca^{2+} influx mediates the EGF-triggered insulin secretion in MIN6 cells. (**A–C**) The MIN6 cells were plated onto glass dishes or 24-well plates and grown for 24 hrs. The cells were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. (**A**) At the end of incubation, the solutions were replaced with fresh KRB containing Fluo-3 AM dissolved (1 mg/ml) in DMSO, and were incubated for 1 hr. At the end of incubation, cells were incubated with none (untreated) or 1 mM EGTA for 30 min., and were then treated with 15 nM EGF. Images were captured on an inverted confocal microscope with a 20(objective lens. The data shown are the mean ± S.E., $n = 7$. (**B**) At the end of incubation, the solutions were replaced with fresh KRB containing none (untreated), 1 mM EGTA or 5 μM BAPTA/AM and were incubated for 30 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. $*P < 0.05$ compared with EGF-treated cells. (**C**) At the end of incubation, the solutions were replaced with fresh KRB containing none (untreated) or 10 μM nifedipine and were incubated for 30 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. $*P < 0.05$ compared with EGF-treated cells. (**B–C**) The data shown are the mean ± S.D. from two independent assays performed in duplicate.

To identify the PLD isozyme responsible for stimulating insulin secretion, we examined the effects of the over-expression and silencing of PLD isozymes. We transfected MIN6 cells with an empty vector, PLD1 or PLD2, and stimulated them with EGF. PLD1 mediated glucose-dependent insulin secretion, as shown previously (data not shown) [32]. In contrast, PLD2 exclusively mediated EGF-dependent insulin secretion, and over-expression of PLD1 showed a limited effect (Fig. 3C). Furthermore, silencing of PLD2, but not PLD1, abolished EGF-induced insulin secretion (Fig. 3E). The same results were also observed in RINm5F cells (data not shown). Finally, EGF-dependent PLD activity, as measured with PBt formation, was modulated exclusively by PLD2 over-expression or silencing (Fig. 3D and F), which suggests that PLD2 is required for EGF-stimulated insulin secretion in these cells.

PLD activity is dependent on Ca^{2+} influx in MIN6 cells

Because both a Ca^{2+} influx and PLD activity are required for EGF-dependent insulin secretion, we analysed the relationship between the two phenomena by testing the effect of the Ca^{2+} influx on PLD activity and the effect of PLD activity on the Ca^{2+} influx. Blocking Ca^{2+} influx by using EGTA or BAPTA/AM inhibited most of the PLD activity (Fig. 4A), which correlated with EGF-dependent insulin secretion (Fig. 2B). However, inhibiting PLD activity by silencing PLD isozymes, which we confirmed through successful silencing of PLDs by Western blotting, had little effect on the EGF-dependent Ca^{2+} influx (Fig. 4B), which suggest that the Ca^{2+} influx is upstream of PLD activation in EGF-dependent insulin secretion.

EGF-stimulated insulin secretion in mouse pancreatic islets requires Ca^{2+} influx and PLD activity

To confirm the physiological significance of EGF, Ca^{2+} and PLD on insulin secretion, we prepared primary cultures of mouse islets. As expected, EGF rapidly increased insulin secretion (Fig. 5A) with a 1-min. treatment. Inhibiting the Ca^{2+} influx or PLD activity completely blocked the EGF-induced insulin secretion (Fig. 5B and C), thus indicating that EGF-stimulated insulin secretion in mouse pancreatic islets requires a Ca^{2+} influx and PLD.

EGF lowers plasma glucose and increases plasma insulin levels

To confirm our *in vitro* findings that EGF could stimulate the release of insulin, we characterized the EGF-mediated responses

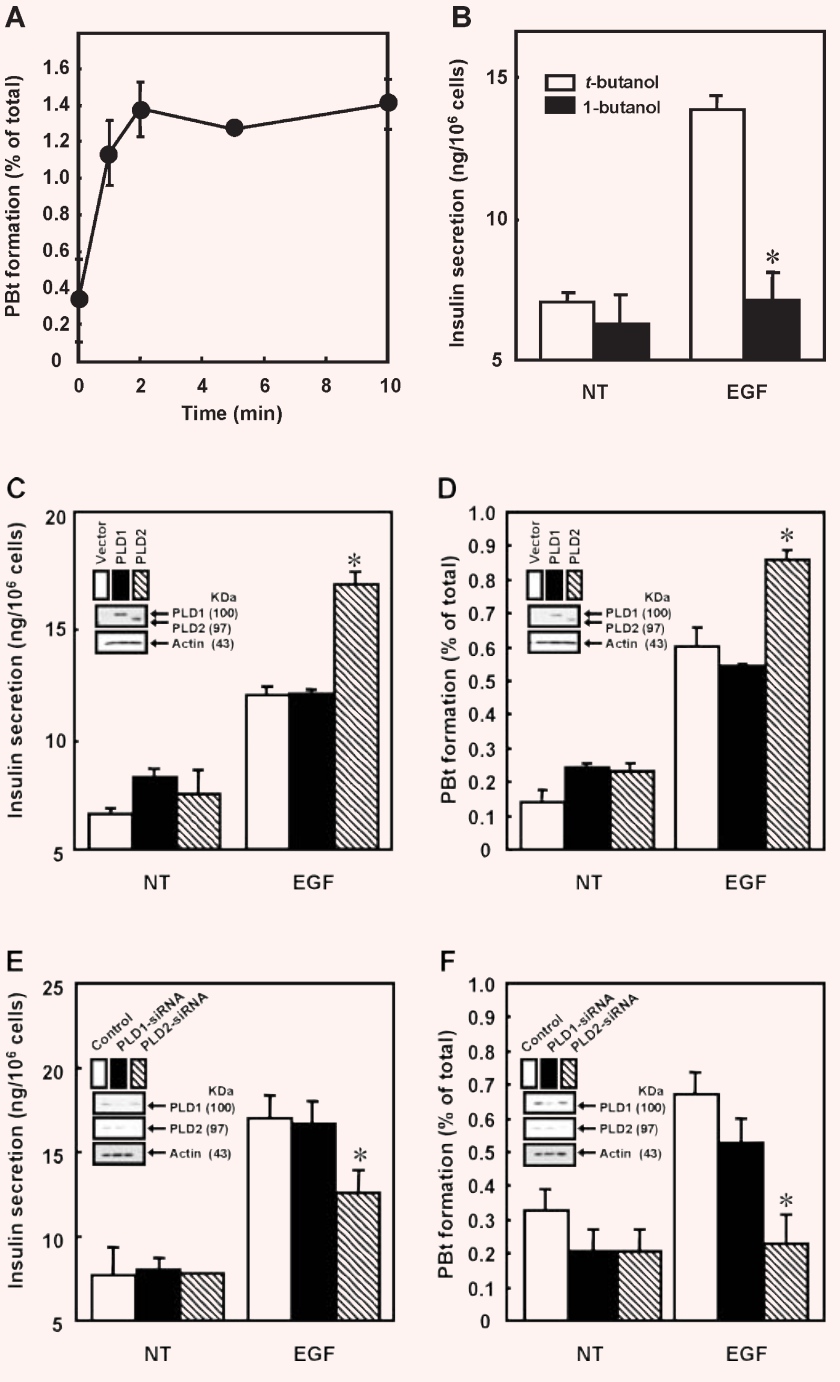

Fig. 3 PLD2 is specifically involved in the EGF-dependent insulin secretion. (**A**) The MIN6 cells were plated onto 6-well plates and grown for 24 hrs. The cells were washed twice with KRB, and were then incubated for 4 hrs at 37°C in the KRB solution in the presence of [^{3}H]myristic acid. At the end of incubation, 15 nM EGF stimulation was performed for the indicated amount of time. The intensities of PBt spots were measured after 0-, 1-, 2-, 5- or 10-min. accumulation in the presence of 1-butanol and EGF. (**B**) The MIN6 cells were plated onto 24-well plates and grown for 24 hrs. The cells were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. At the end of incubation, the solutions were replaced with fresh KRB containing 0.4% t-butanol or 1-butanol and were incubated for 10 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. $*P < 0.05$ compared with EGF-treated cells. (**C–F**) The MIN6 cells were plated onto 24-well plates (for measuring insulin levels) or 6-well plates (for measuring PLD activity) and transfected with the indicated plasmids (vector, PLD1, or PLD2 in Fig. 3C and **D**) or siRNAs (control (luciferase), mouse PLD1 or mouse PLD2 in Fig. 3E and F) grown for 24 or 72 hrs. The efficiencies of transfection were approximately 30–40%. For measuring insulin secretion (Fig. 3C and E), the cells were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. At the end of incubation, the solutions were replaced with fresh KRB containing 15 nM EGF, and were incubated for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. $*P < 0.05$ compared with EGF-treated cells. For measuring PLD activity (Fig. 3D and F), the cells were washed twice with KRB, and were then incubated for 4 hrs at 37°C in the KRB solution in the presence of [3H]myristic acid. At the end of incubation, 15 nM EGF stimulation was performed for 0 or 1 min. The intensities of PBt spots were measured after 1 min. of accumulation in the presence of 1-butanol and EGF. $*P < 0.05$ compared with EGF-treated cells. Cells were lysed with KRB containing 0.1% Triton X-100 and subjected to SDS-PAGE, and were then immunoblotted using anti-PLDs antibody (inner boxes of Fig. 3**C** and **D**) and PLD1 (inner boxes of Fig. 3**E** and **F**), PLD2-specific antibody (inner boxes of Fig. 3**E** and **F**) or actin antibody (inner boxes of Fig. 3**C**–**F**). (**A**–**F**) The data shown are the mean ± S.D. from two independent assays performed in duplicate.

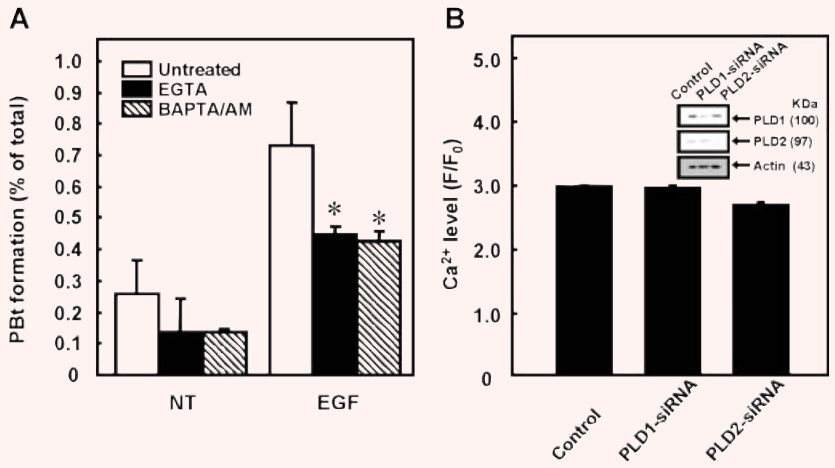

Fig. 4 Ca^{2+} influx is critical for the EGF-induced PLD activation. (**A**) The MIN6 cells were plated onto 6-well plates and grown for 24 hrs. The cells were washed twice with KRB, and were then incubated for 4 hrs at 37°C in the KRB solution in the presence of [^{3}H] myristic acid. At the end of incubation, the solutions were replaced with fresh KRB containing none (untreated), 1 mM EGTA or 5 μM BAPTA/AM and were incubated for 30 min., and were then treated with 15 nM EGF for 0 or 1 min. The intensities of PBt spots were measured after 1 min. of accumulation in the presence of 1-butanol. The data shown are the mean ± S.D. from two independent assays performed in duplicate. $*P < 0.05$ compared with EGF-treated cells. (**B**) The MIN6 cells were plated onto glass dishes and transfected with siRNAs (control [luciferase], mouse PLD1 or mouse PLD2), and were then grown for 24 hrs. The cells were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. At the end of incubation, the solutions were replaced with fresh KRB containing Fluo-3 AM dissolved (1 mg/ml) in DMSO and were incubated for 1 hr. At the end of incubation, cells were treated with 15 nM EGF. Images were captured on an inverted confocal microscope with a 20x objective lens. The data shown are the mean ± S.E. of the peak time, $n = 7$. Cells were lysed with KRB containing 0.1% Triton X-100 and subjected to SDS-PAGE, and were then immunoblotted using anti-PLDs antibody (inner box of Fig. 4B), PLD2-specific antibody (inner box of Fig. 4**B**) or actin antibody (inner box of Fig. 4**B**).

of mouse plasma glucose and insulin levels in normal and obese *db/db* mice by injecting EGF intravenously. In preliminary experiments, EGF (50 μg/kg) achieved a saturated plasma glucose-lowering effect 10 min. after intravenous injection into 7- to 8-week-old male ICR mice (data not shown). This glucose-lowering effect of EGF (50 μg/kg) had a similar potency to insulin and was dose-dependent (Fig. 6A). Moreover, EGF increased plasma insulin levels (Fig. 6B), which suggests that this glucose-lowering effect is due to changes in plasma insulin levels. The kinetics of insulin secretion and changes in glucose were correlated. EGF also reduced plasma glucose in obese *db/db* mice and increased plasma insulin levels (Fig. 6C and D). Taken together, we conclude that EGF has the ability to stimulate insulin secretion and lower plasma glucose in normal and diabetic mice models.

Glucose increases plasma EGF levels

EGF can regulate glucose homeostasis. Therefore, it is possible that the EGF levels in the body can be regulated by the feeding condition. Glucose was injected intravenously into 7- to 8-week-old male ICR mice and the plasma EGF levels were measured (Fig. 7). Twenty minutes after the glucose injection, the plasma EGF levels were elevated. Because glucose stimulates EGF and insulin secretion, it is possible that if insulin level is increased and thereby glucose level is reduced, the opposite effect will be observed on EGF concentration. To test this hypothesis, insulin was injected intravenously 20 min. after the intravenous glucose injection. The results were compared with those from the intravenous saline injection. As a result, the insulin injection significantly attenuated the increase in EGF elevation caused by glucose (Fig. 7). In addition, an oral injection of glucose had the same effect of plasma EGF elevation (data not shown). This suggests that the physiological concentration of EGF can be altered by the feeding conditions, and secreted EGF finally regulates insulin secretion. Although the exact source of secreted EGF is not known, it can be assumed that this increase in plasma EGF induced by the physiological glucose concentration can rapidly mediate the regulation of pancreatic insulin secretion.

Discussion

Insulin synthesis, processing and secretory pathways are highly regulated and dynamic processes. Many factors, including

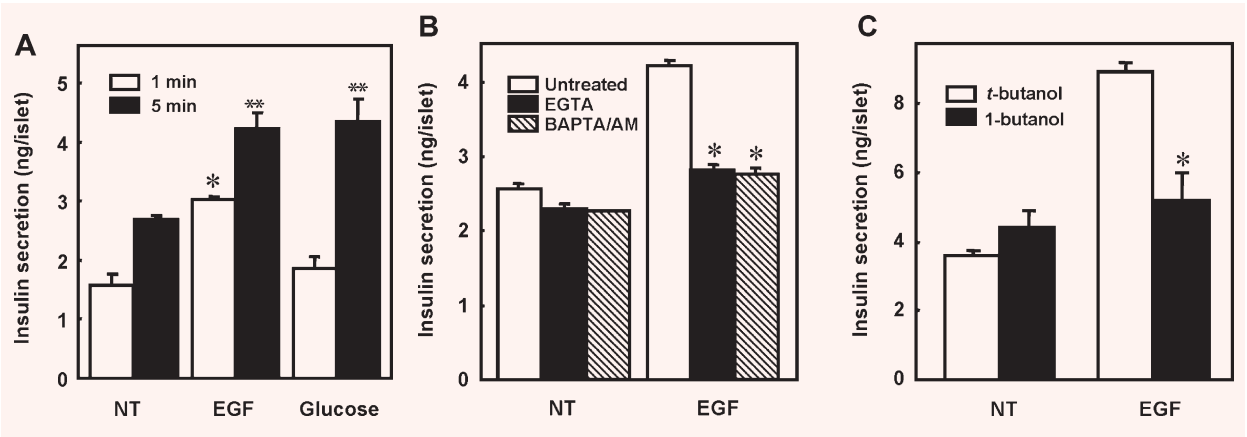

Fig. 5 Insulin secretion is increased by EGF in mouse pancreatic islets through Ca^{2+} influx and PLD activity. (**A–C**) Mouse pancreatic islets were plated onto 12-well plates and grown for 24 hrs. The islets were washed twice with KRB supplemented with 0.2% BSA, and were then incubated for 60 min. at 37°C in the KRB solution. (**A**) At the end of incubation, the solutions were replaced with fresh KRB and were incubated for 1 or 5 min. with none (NT), 15 nM EGF or 11 mM glucose. The incubation medium was sampled for measuring insulin levels. * or **, $P < 0.05$ compared with 1- or 5-min.-treated islets. (**B**) At the end of incubation, the solutions were replaced with fresh KRB containing none (untreated), 1 mM EGTA or 5 μM BAPTA/AM and were incubated for 30 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. *$P < 0.05$ compared with EGF-treated islets. (**C**) At the end of incubation, the solutions were replaced with fresh KRB containing 0.4% of t-butanol and 1-butanol and were incubated for 10 min., and were then treated with 15 nM EGF for 0 or 1 min. The incubation medium was sampled for measuring insulin levels. *$P < 0.05$ compared with EGF-treated islets. (**A–C**) The data shown are the mean ± S.D. from two independent assays performed in duplicate.

nutrients, neurotransmitters and hormones, affect these processes [1, 2]. In this study, for the first time we show that EGF is a novel secretagogue that regulates plasma glucose levels, and determine the mechanism of EGF-induced insulin secretion.

Type 2 diabetes mellitus is characterized by both insulin resistance and impaired insulin secretion. The control of insulin secretion is primarily regulated by glucose itself, but also involves an array of metabolic, neural, hormonal and sometimes pharmacologic factors [1, 33]. Initiators can increase insulin secretion in the absence of other stimulation, but potentiators require the presence of an initiator, usually glucose [1]. Many growth factors are also secreted from the pancreas or affect the pancreas, and their levels are often changed in diabetes [34–36]. However, few studies have been conducted to assess about the role of growth factors in regulating plasma glucose or as insulin secretion initiators. EGF requires only brief exposure (1 min.) to stimulate insulin secretion (Fig. 1A), and increases Ca^{2+} levels when treated alone (Fig. 2A), which indicates that it can function as an initiator. Furthermore, EGF additively stimulates glucose-dependent insulin secretion (Fig. 1D), which means that the EGF effect is glucose-independent. Insulin secretion by glucose has a biphasic pattern, with a peak around 5 min., a nadir at 10 min. and a slowly increasing time-course thereafter. This first phase is key for the insulin-dependent processes that ensure glucose homeostasis [3]. The time-course of EGF receptor-mediated insulin secretion is similar to neurotransmitter release in neuronal cells, and is more rapid than the first phase of glucose-dependent insulin secretion from pancreatic β cells. The EGF-induced release of insulin required a rapid Ca^{2+} influx compared with glucose, which requires 3–4 min. for Ca^{2+} influx, mainly due to the time for glucose metabolism and a delayed change of the ATP/ADP ratio (data not shown). Insulin secretion can sometimes be regulated through classical signalling cascades involving transmembrane receptors, heterotrimeric G-proteins and second messengers [36–38]. Therefore, EGF receptor-mediated regulation of insulin secretion is not unreasonable. Here, we defined a new role for EGF as an initiator of insulin secretion, both *in vitro* and *in vivo*, thus indicating the therapeutic potential of EGF in diabetes.

PLD is activated by EGF stimulation, and this activation is a very rapid process compared to those of other PLD-regulating molecules such as glucose [30, 32]. The mechanism underlying EGF-mediated activation of PLD remains controversial [29–31, 39]. Among the potential mechanisms, EGF-dependent increases in Ca^{2+} activate protein kinase C (PKC) and lead to PLD activation [40]. Another report suggested that a Ca^{2+} influx is associated with the activation of PLD, and that PKC is involved in this process [41]. However, there have been limited studies related to the Ca^{2+}-mediated PLD activation of specific isozymes. In the present study, we identified PLD2 as a Ca^{2+}-dependent isozyme in the pancreatic β cells using EGF treatment (Fig. 3C–F). Although PLD1 and 2 share a sequence homology of around 50% and contain similar regulatory domains [20], they show differences in localization and regulatory protein interactions [39, 42]. A previous report suggested that PLD1 and PLD2 regulate different

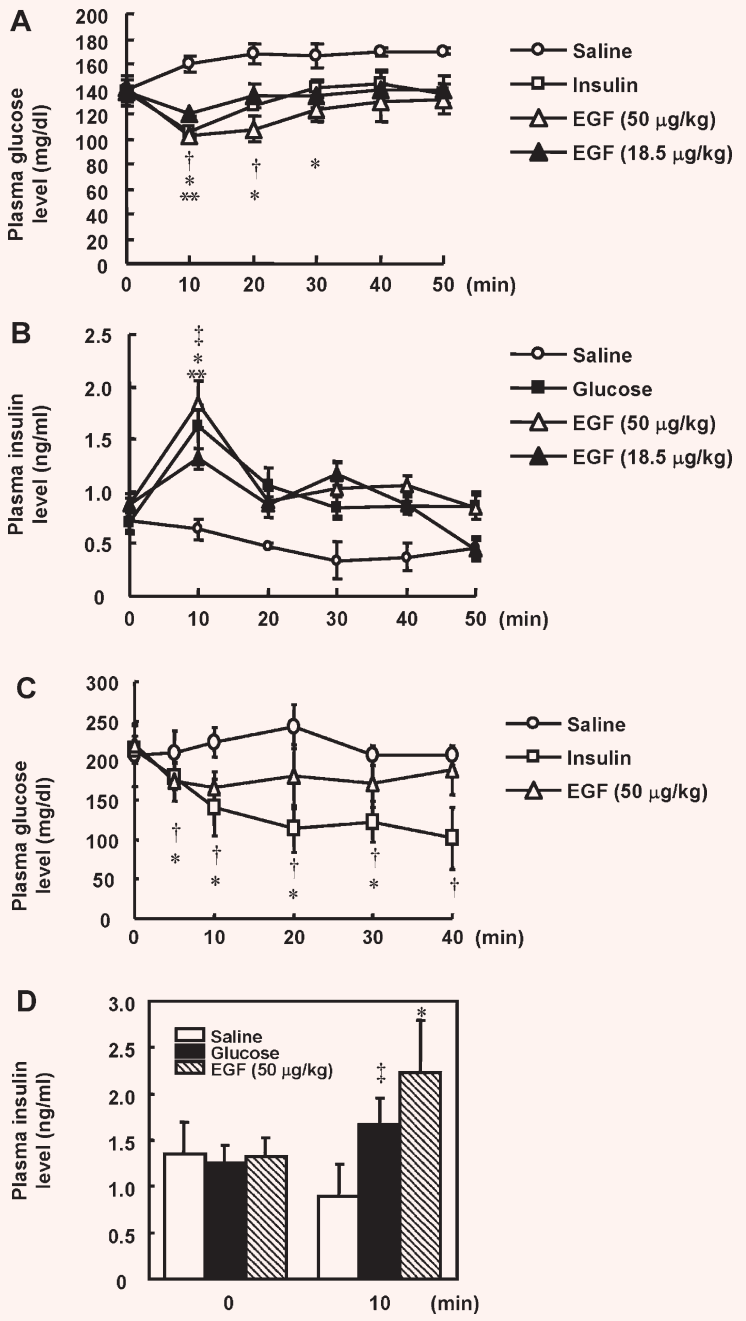

Fig. 6 EGF lowers plasma glucose and increases plasma insulin levels. (**A**) Seven- to 8-week-old male ICR mice (10 mice/group) received intravenous injections of saline (0.9% NaCl in double-distilled water), insulin (0.06 U/kg) or EGF (18.5 or 50 μg/kg), and plasma glucose levels were subsequently measured. † (insulin), * (EGF 50 μg/kg) or ** (EGF 18.5 μg/kg), $P < 0.05$ compared with saline-treated mice in the indicated amount of time. (**B**) Seven- to 8-week-old male ICR mice (10 mice/group) received intravenous injections of saline, glucose (0.5 g/kg) or EGF (18.5 or 50 μg/kg), and plasma insulin levels were subsequently measured. ‡ (glucose), * (EGF 50 μg/kg) or ** (EGF 18.5 μg/kg), $P < 0.05$ compared with saline-treated mice in the indicated amount of time. (**C**) Obese C57BLKSJ-*db/db* mice (six mice per group) received intravenous injections of saline (0.9% NaCl in double-distilled water), insulin (0.06 U/kg) or EGF (50 μg/kg), and plasma glucose levels were subsequently measured. † (insulin) or * (EGF 50 μg/kg), $P < 0.05$ compared with saline-treated mice in the indicated amount of time. (**D**) Obese C57BLKSJ-*db/db* mice (six mice per group) received intravenous injections of saline, glucose (0.5 g/kg) or EGF (50 μg/kg), and plasma insulin levels were subsequently measured. Plasma insulin levels before and 10 min. after injection were compared. ‡ (glucose) or * (EGF 50 μg/kg), $P < 0.05$ compared with saline-treated mice in the indicated amount of time. All animals had free access to water. Animal care was conducted in accordance with the guidelines of our institution. (**A–D**) The data shown are mean ± S.E.

phases of exocytosis in mast cells via a two-step process [21]: translocation of granules to the cell periphery, regulated by granule-associated PLD1, and a Ca^{2+}-dependent fusion of granules with the plasma membrane, regulated by plasma membrane–associated PLD2. Differently from the previous report suggesting PLD1 as a mediator of glucose-stimulated insulin secretion [32], in our hands, EGF stimulation required the activation of PLD2. The specific activations of PLD1 by glucose and of PLD2 by EGF have different kinetics, and their mechanisms require further clarification. We detected PLD2 in MIN6 cells, both by Western blotting using a PLD2-specific antibody (Fig. 3C–F) and by RT-PCR (data not shown). Furthermore, we used over-expression and

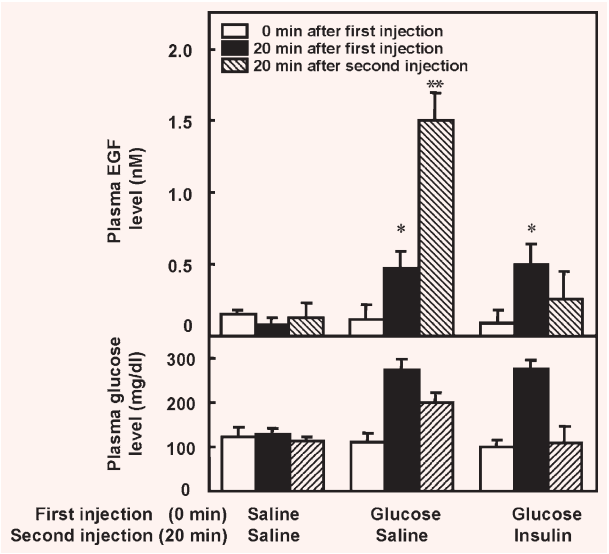

Fig. 7 Glucose increases plasma EGF levels. Seven- to 8-week-old male ICR mice (seven mice per group) received intravenous injections of saline or glucose (2 g/kg) for a first injection (0 min.), and saline or insulin (0.06 U/kg) for a second injection (20 min.). As indicated, group and plasma EGF and glucose levels were subsequently measured. The data shown are the mean ± S.E., * or ** $P < 0.05$ compared with saline-treated mice in the indicated amount of time. All animals had free access to water. Animal care was conducted in accordance with the guidelines of our institution.

silencing strategies to determine that PLD2, not PLD1, mediated EGF-dependent insulin secretion (Fig. 3C and E). These results suggest that glucose stimulates insulin secretion via PLD1 with a relatively late time course, whereas EGF activates plasma membrane–localized PLD2, leading to rapid fusion of pre-docked insulin granules with the plasma membrane. Our work supports the notion that PLD1 and PLD2 mediate different pathways for regulating insulin secretion. Since PLDs are important molecules in exocytotic processes, the investigation of PLDs will provide significant insight into the regulatory mechanisms of insulin secretion.

Metabolism of glucose results in the closure of ATP-sensitive K^+ channels, and the subsequent plasma membrane depolarization opens voltage-sensitive Ca^{2+} channels [43]. The resultant rise in the cytoplasmic free Ca^{2+} concentration is both necessary and sufficient for triggering an initial phase of insulin release that is mediated by the fusion of pre-docked insulin granules with the plasma membrane [38]. Our results show that an EGF-stimulated Ca^{2+} influx mediates insulin secretion, especially through L-type Ca^{2+} channels (Fig. 2B and C). EGF was reported to regulate store-operated Ca^{2+} channel activation, as well as Ca^{2+} release from stores. Previous studies suggest that proliferative responses to EGF are dependent on the cellular influx of Ca^{2+} through Ca^{2+}-selective channels [44, 45]. In the present work, we determined that EGF-stimulated insulin secretion required both Ca^{2+} influx and PLD activity (Figs. 2B and 3B). Our findings (Fig. 4) suggest that the Ca^{2+} influx is an upstream signal for PLD activity. A previous report has indicated that secretory stimuli activate PLD activity in pancreatic β cells [32]: treatment with channel blockers profoundly decreased insulin secretion from MIN6 cells, as well as the cellular PLD activity, in the presence of glucose. Accordingly, our results strongly indicate a close relationship between a secretagogue-induced Ca^{2+} influx and PLD activity in insulin secretion by pancreatic β cells.

EGF regulates pancreatic function and is produced in the pancreas and pancreatic juice [6, 46]. EGFR is expressed throughout the human foetal pancreas, and mice lacking EGFR showed abnormal formation of pancreatic islets [12]. Some members of the EGF family play a role in the development of the pancreas [11]. EGF regulates the insulin content of rat pancreatic β cells as well as their regeneration [13–15]. Furthermore, EGF deficiency is associated with diabetes mellitus; in diabetic animals, EGF or EGFR levels are decreased in various organs or fluids, such as the liver, the submandibular gland, plasma and milk [7, 16, 47, 48]. Interestingly, levels of these proteins often recover after insulin curative treatment [48], and EGF and insulin act synergistically during diabetic healing [49]. A recent report suggested that EGF can regulate glucose homeostasis [50]. They focused on the role of EGF in regulating the plasma glucagon levels depending on the presence of glucose, and observed this effect using an animal model. Furthermore, combination therapy with EGF and gastrin induced β-cell regeneration in rodents with chemically induced diabetes [15]. Therefore, this study focused on the plasma insulin levels based on the results from insulinoma cell lines and islets, and determined the mechanism of EGF-dependent insulin secretion. Consistent with our *in vitro* findings from MIN6 (Fig. 1), RINm5F (data not shown) cell line and mouse pancreatic islets (Fig. 5), which indicated that EGF could stimulate the release of insulin, we found that EGF increased plasma insulin levels and decreased plasma glucose levels in normal and even in diabetic mice (Fig. 6). Furthermore, we observed that physiological EGF levels were increased by a glucose injection and decreased after a sequential insulin injection (Fig. 7). While the effect of insulin to reduce the EGF levels after the glucose injection may indeed be secondary to the reduction of blood glucose, it is also possible that insulin could suppress EGF through a direct mechanism. From these results, we speculate that physiological EGF rapidly increases insulin secretion, and this process might be important in short-term regulation of plasma glucose levels. Although the exact source of secreted EGF is unknown, it can be assumed that this increase in plasma EGF induced by the physiological glucose concentration can rapidly mediate pancreatic insulin secretion. Further studies will be needed to determine which pancreatic cells secrete EGF and how this secretion is regulated. It is likely that EGF-dependent insulin secretion plays a similar function to that of glucose on glucose homeostasis in our body. Reducing the endogenous level of EGF using knock-down, antibody, or aptamer would indicate the physiological function of EGF on glucose and insulin homeostasis. Taken together, our observations on the role of EGF on insulin secretion and β-cell regeneration may contribute

to a better understanding of the pathophysiology of diabetes mellitus, in which serum EGF levels are diminished. Furthermore, the effect of EGF in diabetic mice indicates the potential usefulness of EGF as a potential treatment of diabetes.

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