Acute stimulation of glucagon secretion by linoleic acid results from GPR40 activation and \([\text{Ca}^{2+}]_{i}\) increase in pancreatic islet \(\alpha\)-cells

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Abstract

The role of free fatty acids (FFAs) in glucagon secretion has not been well established, and the involvement of FFA receptor GPR40 and its downstream signaling pathways in regulating glucagon secretion are rarely demonstrated. In this study, it was found that linoleic acid (LA) acutely stimulated glucagon secretion from primary cultured rat pancreatic islets. LA at 20 and 40 \(\mu\)mol/l dose-dependently increased glucagon secretion both at 3 mmol/l glucose and at 15 mmol/l glucose, although 15 mmol/l glucose reduced basal glucagon levels. LA at 20 and 40 \(\mu\)mol/l dose-dependently increased glucagon secretion both at 3 mmol/l glucose and at 15 mmol/l glucose, although 15 mmol/l glucose reduced basal glucagon levels. LA induced an increase in cytoplasmic free calcium concentrations (\([\text{Ca}^{2+}]_{i}\)) in identified rat \(\alpha\)-cells, which is reflected by increased Fluo-3 intensity under confocal microscopy recording. The increase in \([\text{Ca}^{2+}]_{i}\) was partly inhibited by removal of extracellular \(\text{Ca}^{2+}\) and eliminated overall by further exhaustion of intracellular \(\text{Ca}^{2+}\) stores using thapsigargin treatment, suggesting that both \(\text{Ca}^{2+}\) release and \(\text{Ca}^{2+}\) influx contributed to the LA-stimulated increase in \([\text{Ca}^{2+}]_{i}\) in \(\alpha\)-cells. Double immunocytochemical stainings showed that GPR40 was expressed in glucagon-positive \(\alpha\)-cells. LA-stimulated increase in \([\text{Ca}^{2+}]_{i}\) was blocked by inhibition of GPR40 expression in \(\alpha\)-cells after GPR40-specific antisense treatment. The inhibition of phospholipase C activity by U73122 also blocked the increase in \([\text{Ca}^{2+}]_{i}\) by LA. It is concluded that LA activates GPR40 and phospholipase C (and downstream signaling pathways) to increase \(\text{Ca}^{2+}\) release and associated \(\text{Ca}^{2+}\) influx through \(\text{Ca}^{2+}\) channels, resulting in increase in \([\text{Ca}^{2+}]_{i}\) and glucagon secretion. *Journal of Endocrinology* (2011) 210, 173–179

Introduction

Pancreatic islet dysfunction is a major pathophysiological change in type 2 diabetes that is characterized by high blood glucose level (Spellman 2007). The \(\beta\)-cell dysfunction leads to unsatisfied compensation to the development of insulin resistance, causing an increase in blood glucose level (Weir & Bonner-Weir 2004, Prentki & Nolan 2006, Wajchenberg 2007). The islet \(\alpha\)-cell also regulates blood glucose level by secreting glucagon, which activates gluconeogenesis to increase blood glucose level (Gerich 1981, Gromada et al. 2007, Quesada et al. 2008). It has been reported that \(\alpha\)-cell dysfunction also occurred during type 2 diabetes. For example, the inhibition of glucagon secretion after feeding was diminished in patients of type 2 diabetes (Reaven et al. 1987, Mitrakou et al. 1992, Larsson & Ahren 2000). The increase in glucagon level may contribute to the deterioration of high blood glucose level in type 2 diabetes (Unger & Orci 1977, Shah et al. 2000).

The levels of free fatty acids (FFAs) elevate in type 2 diabetes and lipotoxicity is considered as an important cause of \(\beta\)-cell dysfunction (Poitout & Robertson 2002, Wilding 2007, Cnop 2008). On the other hand, it has been reported that FFAs stimulated glucagon secretion, leading to a possible \(\alpha\)-cells dysfunction with an increased glucagon secretion in type 2 diabetes (Bollheimer et al. 2004, Hong et al. 2005, Fujiwara et al. 2007). It is known now that FFAs not only are the fuel for cell metabolism but also function as extracellular messengers to activate intracellular signal pathways through membrane receptors such as GPR40 (Salehi et al. 2005, Nolan et al. 2006, Meidute Abraviciene et al. 2008). GPR40 was firstly found to mediate FFA-stimulated insulin secretion (Itoh et al. 2003, Fujiwara et al. 2005). GPR40 was also reported to mediate FFA-stimulated glucagon secretion (Flodgren et al. 2007). The intracellular mechanism underlying stimulated glucagon secretion by FFAs remains unknown. In this study, we observed the effects of linoleic acid (LA) on glucagon secretion via GPR40 activation, downstream phospholipase C (PLC) signaling system, and the increase in \([\text{Ca}^{2+}]_{i}\) in primary cultured rat pancreatic \(\alpha\)-cells.
Materials and Methods

Chemicals

LA (water-soluble), histopaque-1077, dispase, collagenase (type V), DNase I, BSA, RPMI-1640, thapsigargin and all reagents for experimental solutions were purchased from Sigma. U73122 was obtained from Calbiochem (San Diego, CA, USA). Fluo-3/AM was purchased from Invitrogen. FCS, HEPES and penicillin/streptomycin were from Gibco. GPR40 polyclonal antibody and the secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Glucagon monoclonal antibody was purchased from Sigma.

Preparation and culture of rat pancreatic islet cells

Pancreatic islets were isolated from 10- to 12-week-old male Sprague–Dawley rats as described previously (Zhao et al. 2008). Briefly, rats were killed by CO₂ inhalation and the pancreas was inflated by injecting 10 ml collagenase solution into it through the bile duct. The collagenase solution was composed of 0.5 mg/ml collagenase, 0.1 mg/ml DNase I, and 1 mg/ml BSA in Hank’s balanced salt solution (HBSS). The pancreas was isolated and digested at 37 °C for 30 min, and then dispersed by shaking. The islets were separated by histopaque-1077 density gradient centrifugation and collected for cell isolation. The islets were dispersed into single cells by digestion with dispase solution. Dispase solution was composed of 1 mg/ml dispase, 0.1 mg/ml DNase I, and 1 mg/ml BSA in Ca²⁺-free HBSS. The islet cells were plated onto glass cover slips coated with 0.01% poly-l-lysine and then cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed every 2 days. The experiments were performed during days 3–6 in culture.

Measurement of [Ca²⁺]i, in single α-cells

Islet cells were loaded with 1 µM Fluo-3/AM in RPMI-1640 medium for 30 min at 37 °C. Cells were subsequently rinsed with bath solution and kept for 20 min in this solution to allow full de-esterification of the dye and to tranquilize the cells. The intensities of the cells were recorded and analyzed using Olympus FV-1000 confocal microscopy system (Zhao et al. 2003). The following fluorescence filters were used for Fluo-3 imaging: excitation laser of 480 nm and emission filter of 510 nm. The cells were imaged over time, and the changes in fluorescence intensity of Fluo-3 that reflect calcium changes were constructed from the series of X–Y images using Olympus FV-1000 software (Olympus, Tokyo, Japan). All calcium imaging data were presented as a ratio of fluorescence over baseline fluorescence (F/F₀) and plotted over time. Cells were constantly perfused at a rate of 3 ml/min. Experimental reagents were dissolved in the bath solution just before the recordings and delivered through the perfusion system. The α-cells were identified by immunocytochemical staining using the glucagon antibody after recording. The normal bath solution used for [Ca²⁺]i measurements was composed of (mM): 140 NaCl, 4-7 KCl, 2-6 CaCl₂, 1.2 MgSO₄, 1 NaHCO₃, 1.2 Na₂HPO₄, 3 glucose, and 5 HEPES (pH 7.4 with NaOH).

Glucagon secretion assay

Rat pancreatic islets were cultured in 10% RPMI overnight. For glucagon secretion test, the islets were preincubated with Hanks solution with 0.1% BSA for 30 min at 37 °C. The buffer was then changed, and the islets were incubated at 3 mmol/l glucose or 15 mmol/l glucose with or without LA in Hanks solution for 15 min at 37 °C. Each incubation vial contained 50 islets in 0.5 ml of buffer solution. Aliquots of the media were collected after incubation and frozen for the subsequent glucagon assay. Glucagon levels in media were assayed using glucagon ELISA kits according to the instruction (Wako Chemicals, Richmond, VA, USA).

Immunocytochemical staining

For immunocytochemical staining of GPR40 and glucagon, the cells were cultured on cover slips treated with poly-l-lysine. After 4 days growth, the cells were fixed by 4% parafomaldehyde for 10 min at room temperature (RT). The following steps were done in series for the staining: Triton X-100 (0.1%) in 10% blocking serum was applied for 30 min at RT; the polyclonal GPR40 antibody originated from rabbit was applied overnight at RT; the biotinylated secondary antibody (goat anti-rabbit antibody) was applied for 1 h at RT. Avidin/biotin complex (ABC) solution was applied for 1 h at RT; DAB-hydrogen peroxide solution was applied; the reaction was stopped by transferring the cover slips to distilled water. The cover slips were washed carefully with PBS containing 0.1% Triton X-100 (three times, 5 min per time) between each two steps. After GPR40 staining, the cover slips with staining cells were incubated in series with monoclonal glucagon antibody for 30 min at RT, FITC-conjugated secondary antibody for 30 min in dark at RT. The cover slips were washed carefully with PBS containing 0.1% Triton X-100 (three times, 5 min per time) between each two steps. The glucagon-positive cells were observed using microscopy with excitation wavelength of 450 nm and the GPR40-positive cells were observed using normal light. The overlay of GPR40-positive cells with glucagon-positive cells was analyzed to clarify the distribution of GPR40 in α-cells. For identification of α-cells after [Ca²⁺]i recording, the cells were fixed and blocked and then treated in series with monoclonal glucagon antibody, biotinylated secondary antibody, ABC complex, DAB-hydrogen peroxide solution. The cover slips were washed carefully with PBS containing 0.1% Triton X-100 (three times, 5 min per time) between each two steps. Photos of the cells were taken which were compared with images of confocal recording of [Ca²⁺]i, to identify α-cells in the recording.
Antisense intervention

Pancreatic islet cells were cultured for 2 days in RPMI-1640 supplemented with 10% FCS and then treated with the media in the presence of either GPR40-specific morpholino antisense oligonucleotide or M40 a nonspecific random-sequence morpholino oligonucleotide as control for 2 days (Salehi et al. 2005). The morpholinos were delivered into the cells using the manufacturer’s special delivery system according to the instruction (Gene Tools, Philomath, OR, USA). The cells were then used for [Ca\textsuperscript{2+}] recording and immunocytochemical recording.

Statistical analysis

The data are represented as mean±S.E.M. for each group. One-way ANOVA was used to analyze the statistical significance between different groups. \(P<0.05\) was taken as the minimum level of significant difference.

Results

LA-stimulated glucagon secretion from rat islets

Isolated rat islets were incubated with various concentrations of LA under static conditions. In Fig. 1, addition of LA in incubation medium for 15 min dose-dependently increased glucagon secretion at 20 and 40 \(\mu\text{mol/l}\) in the presence of 3 mmol/l glucose \((P<0.01, n=12)\). In the high concentration of glucose at 15 mmol/l, glucagon secretion was significantly decreased compared with 3 mmol/l glucose \((P<0.05, n=12)\), whereas LA still significantly increased glucagon secretion in a comparable and dose-dependent manner \((P<0.01, n=12)\).

The effects of LA on [Ca\textsuperscript{2+}]\textsubscript{i} in \(\alpha\)-cells

The [Ca\textsuperscript{2+}]\textsubscript{i} was measured using confocal microscopy, and the type of the cells recorded was identified after recording by immunocytochemical staining. Photos of the recorded cells were taken under different conditions. The cells that were loaded with Fluo-3 under the laser of 480 nm excitation light (A), and cells recorded were stained by glucagon antibodies. The arrow indicates a glucagon-positive cell (B). LA-stimulated significant increase in [Ca\textsuperscript{2+}]\textsubscript{i}, in \(\alpha\)-cells at 3 mmol/l glucose (C) and at 15 mmol/l glucose (D).

The sources of calcium for [Ca\textsuperscript{2+}]\textsubscript{i}, response of \(\alpha\)-cells to LA

The increase in [Ca\textsuperscript{2+}]\textsubscript{i} is the result of calcium release from either intracellular calcium stores or extracellular calcium influx, or both. In the calcium-free solution, LA stimulated increase in [Ca\textsuperscript{2+}]\textsubscript{i}, but the second phase of increase in [Ca\textsuperscript{2+}]\textsubscript{i} was reduced (Fig. 3A). In the normal calcium-containing solution, intracellular calcium stores were exhausted by preincubating the cells with thapsigargin for 30 min. Under such condition, LA did not induce any increase in [Ca\textsuperscript{2+}]\textsubscript{i} in \(\alpha\)-cells (Fig. 3B).
The involvement of GPR40 in LA-stimulated increase in \([\text{Ca}^{2+}]_i\) in \(\alpha\)-cells

The expression of FFA membrane receptors in primary cultured rat islet \(\alpha\)-cells was identified. It was found that glucagon-containing \(\alpha\)-cells were GPR40 staining-positive via immunocytochemical double staining. A representative staining result of \(\alpha\)-cells was shown in Fig. 4A. To further confirm whether the increase in \([\text{Ca}^{2+}]_i\), was mediated by the activation of GPR40, we blocked the expression of GPR40 using GPR40 morpholino antisense oligonucleotide. The \(\alpha\)-cells that were treated with a nonsense morpholino did not show a significant difference in the immunostaining of GPR40 and \([\text{Ca}^{2+}]_i\), response to LA compared with the \(\alpha\)-cells that were untreated. After specific antisense oligonucleotide treatment, the staining of GPR40 in \(\alpha\)-cells became significantly weakened compared with the \(\alpha\)-cells in control (Fig. 4B). Antisense oligonucleotide-treated islet \(\alpha\)-cells had a significantly reduced \([\text{Ca}^{2+}]_i\), response to LA stimulation compared with the control cells treated with nonspecific random-sequence morpholino oligonucleotides (Fig. 4C and D).

Involvement of PLC in LA-stimulated increase in \([\text{Ca}^{2+}]_i\) in \(\alpha\)-cells

It was reported that GPR40 activation is linked to the activation of PLC, which leads to the production of inositol triphosphate (IP₃) and diacylglycerol (DAG; Salehi et al. 2005, Meidute Abaraviciene et al. 2008). In this study, we showed that inhibition of PLC activities by U73122 totally blocked the increase in \([\text{Ca}^{2+}]_i\), by LA (Fig. 5). The negative control of U73122, U73133, did not show any blocking effect on LA-stimulated increase in \([\text{Ca}^{2+}]_i\), in \(\alpha\)-cells.

The involvement of increase in \([\text{Ca}^{2+}]_i\) in LA-stimulated glucagon secretion

After exhausting thapsigargin-sensitive intracellular calcium stores by thapsigargin pretreatment, \(\alpha\)-cells did not show any increase in \([\text{Ca}^{2+}]_i\) by LA as shown (Fig. 3), and there was no increase in glucagon secretion in response to LA either (Fig. 6).

Discussion

In this study, we first demonstrated the stimulatory effects of LA on glucagon secretion from rat islets in vitro. Currently, the relationship between FFAs and glucagon secretion is unclear, and several contradictory reports have been published. The inhibitory actions of FFAs on glucagon secretion in humans and in perfused rat pancreas were reported earlier (Gerich et al. 1976a,b, Campillo et al. 1979). Recent studies have shown that FFAs had a stimulatory effect on glucagon secretion (Bollheimer et al. 2004, Olofsson et al. 2005).
demonstrated that the increase in $[\text{Ca}^{2+}]_{i}$ can be divided into two phases: the first phase is a transient peak increase and the second phase is a plateau with some oscillations. Without external $\text{Ca}^{2+}$, LA also stimulated increase in $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells, but the second phase was significantly reduced or totally abolished. Therefore, it is suggested that the first phase of increase in $[\text{Ca}^{2+}]_{i}$ results from $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores whereas the second phase of increase results from influx of extracellular $\text{Ca}^{2+}$. In a separate experiment, exhaustion of intracellular $\text{Ca}^{2+}$ stores using thapsigargin totally eliminated LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells, indicating that $\text{Ca}^{2+}$ release from thapsigargin-sensitive $\text{Ca}^{2+}$ stores is not only responsible for the initiation first phase of $[\text{Ca}^{2+}]_{i}$ increase but also for triggering the increase in $\text{Ca}^{2+}$ influx that forms the second phase of $[\text{Ca}^{2+}]_{i}$ increase in $\alpha$-cells. The specific channels responsible for $\text{Ca}^{2+}$ release and $\text{Ca}^{2+}$ influx remain to be elucidated. According to the dependence of $\text{Ca}^{2+}$ influx in the second phase on the $\text{Ca}^{2+}$ release in the first phase, we suggest that store-operated $\text{Ca}^{2+}$ entry is responsible for the $\text{Ca}^{2+}$ influx during the stimulation by LA in $\alpha$-cells (Rosado et al. 2005, Parekh 2006).

The signaling pathway mediating FFA-stimulated increase in $[\text{Ca}^{2+}]_{i}$ was elucidated in this study. FFAs not only are metabolized to generate energy but also activate specific G-protein-coupled receptors such as GPR40 and the downstream intracellular signaling molecules (Itoh et al. 2003, Shapiro et al. 2005, Meidute Abraviciene et al. 2008). Although it was first found in $\beta$-cells, GPR40 was reported to be expressed in $\alpha$-cells (Flodgren et al. 2007). In this study, it is confirmed that GPR40 is expressed in $\alpha$-cells using double immunocytochemical staining. When GPR40 expression was inhibited by RNA interference, LA failed to increase $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells, suggesting that GPR40-mediated

**Figure 5** Involvement of PLC in LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells. (A) LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$, in control $\alpha$-cells. (B) LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$, in the $\alpha$-cells treated by $1 \mu \text{M} \text{U73122}$, the negative control of U73132. (C) U73122 ($1 \mu \text{M}$ for $10 \text{ min treatment}$) totally blocked LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$, in $\alpha$-cells.

2004). Palmitate and oleate stimulated glucagon secretion from rat islets in vitro (Bollheimer et al. 2004, Olofsson et al. 2004). A spectrum of medium to long-chain FFAs has also been shown to enhance glucagon secretion from isolated mouse islets (Hong et al. 2005). The stimulatory effects of FFAs on glucagon secretion were also supported by other reports (Flodgren et al. 2007, Fujiwara et al. 2007). Our present results are in accordance with these studies, suggesting that FFAs stimulate glucagon secretion.

The mechanism behind the stimulatory effects of FFAs on glucagon secretion has not been elucidated. In this study, we demonstrated that the increase in $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells is a direct trigger for FFA-induced glucagon secretion. The level of $[\text{Ca}^{2+}]_{i}$ is a critical factor controlling hormone secretion in most endocrine cells (Cremona et al. 2004, Rutter et al. 2006). Glucagon secretion is also initiated by an increase in $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells, and many stimuli known to increase glucagon secretion are associated with elevated $[\text{Ca}^{2+}]_{i}$ in $\alpha$-cells (Johansson et al. 1987, Gromada et al. 1997, Barg et al. 2000). In this study, we observed a significant increase in $[\text{Ca}^{2+}]_{i}$ in rat $\alpha$-cells when they were acutely stimulated by LA. LA-induced increase in $[\text{Ca}^{2+}]_{i}$ can be blocked by prior exhaustion of intracellular $\text{Ca}^{2+}$ stores using thapsigargin. After pretreatment with thapsigargin, LA did not stimulate glucagon secretion from $\alpha$-cells, which was paralleling the elimination of LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$ in these cells. These results strongly support that LA acutely stimulates glucagon secretion by elevating $[\text{Ca}^{2+}]_{i}$ levels in $\alpha$-cells.

Increase in $[\text{Ca}^{2+}]_{i}$ can be achieved through mobilization of both internal and external sources of calcium (Carafoli 1988, Sayer 2002). In this study, it was shown that LA-stimulated increase in $[\text{Ca}^{2+}]_{i}$ resulted from both $\text{Ca}^{2+}$ release from intracellular IP$_3$-sensitive $\text{Ca}^{2+}$ stores and $\text{Ca}^{2+}$ entry through membrane $\text{Ca}^{2+}$ channels from extracellular

**Figure 6** The involvement of increase in $[\text{Ca}^{2+}]_{i}$ in LA-stimulated glucagon secretion. After exhausted thapsigargin-sensitive intracellular calcium stores by thapsigargin pretreatment, there was no increase in glucagon secretion in response to LA (NSD, no significant difference, $n=8$).
LA-stimulated increase in \( [Ca^{2+}] \), in these cells. It is reported that GPR40 is linked to PLC and downstream signaling, such as IP3 and protein kinase C (Fujiwara et al. 2005, Shapiro et al. 2005). In this study, inhibition of PLC by U73122 blocked the stimulation of LA on increase in \( [Ca^{2+}] \), in \( \alpha \)-cells. It further supports that the effects of LA are mediated by plasma membrane receptor that linked to PLC.

It is well known that PLC catalyzes PIP2 to generate IP3 and protein kinase C (Fujiwara et al. 1990, Berridge 2009). Taken together, it is concluded that LA activates GPR40 and PLC to generate signaling molecules to induce Ca\(^{2+}\) release and subsequent Ca\(^{2+}\) influx, resulting in increase in \( [Ca^{2+}] \), and glucagon secretion.

The interaction between FFAs and islet endocrine function has been well studied in \( \beta \)-cells, and it is suggested that increased levels of circulating FFAs may contribute to \( \beta \)-cell dysfunction and abnormal insulin secretion in type 2 diabetes (Wilding 2007, Cnop 2008). Type 2 diabetes is characterized not only by abnormal insulin secretion but also by elevated glucagon secretion (Rice et al. 1987, Mitrakou et al. 1992, Larsson & Ahren 2000, Shah et al. 2000). Together with previous studies from different groups, this study further supports that FFAs take part in the development of type 2 diabetes by stimulating glucagon secretion and deteriorating hyperglycemia.

This study underlines the importance of GPR40 in islet dysfunction. Although there are reports suggesting that GPR40 activation may stimulate insulin secretion, it is also suggested that ablation of GPR40 action is protective to many aspects of type 2 diabetes (Steneberg et al. 2005). The involvement of GPR40 in stimulation of glucagon secretion adds more information to the roles of GPR40 in the development of type 2 diabetes. It is indicated that inhibition of GPR40 action may be beneficial to improve hyperglycemia in type 2 diabetes. Nevertheless, further research into GPR40 in diabetes is warranted.

**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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