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Biochemical and Biophysical Research Communications 354 (2007) 240–245
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GPR40 is expressed in glucagon producing cells and affects glucagon secretion

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Received 22 December 2006
Available online 3 January 2007

Abstract

The free fatty acid receptor, GPR40, has been coupled with insulin secretion via its expression in pancreatic β-cells. However, the role of GPR40 in the release of glucagon has not been studied and previous attempts to identify the receptor in α-cells have been unfruitful. Using double-staining for glucagon and GPR40 expression, we demonstrate that the two are expressed in the same cells in the periphery of mouse islets. In-R1-G9 hamster glucagonoma cells respond dose-dependently to linoleic acid stimulation by elevated phosphatidyl inositol hydrolysis and glucagon release and the cells become increasingly responsive to fatty acid stimulation when overexpressing GPR40. Isolated mouse islets also secrete glucagon in response to linoleic acid, a response that was abolished by antisense treatment against GPR40. This study demonstrates that GPR40 is present and active in pancreatic α-cells and puts further emphasis on the importance of this nutrient sensing receptor in islet function.

Keywords: GPR40; Free fatty acid; α-Cell; Glucagon; Pancreatic islets; Diabetes

Previous observations have shown that free fatty acids (FFAs) are not only important metabolites in the process of energy generation and storage, but also function as signaling molecules [1–3]. In a recent study of the effects of FFAs on insulin secretion, we found that FFAs exert the stimulatory effect on the β-cell by hormone like properties, i.e., activating the G-protein coupled receptor 40 (GPR40) [4]. Such a modulatory effect of FFAs, through the GPR40 pathway, on insulin secretion has also been reported by other research groups [2,5]. GPR40 is markedly expressed in pancreatic β-cells, whereas so far no expression has been reported in α-cells. Upon stimulation by medium to long chain FFAs, GPR40 activation results in elevation of $[Ca^{2+}]_i$ both via IP_3-mediated release from the ER and via influx through L-type Ca^{2+}-channels [6]. Although FFAs are vital components involved in the regulation of many metabolic processes, the activation of GPR40 seems to be of great importance for acute insulin secretion. FFAs have been shown to acutely stimulate glucagon secretion [7–9], but the mechanisms behind this are not fully understood. In the present work, we studied the role of GPR40 in FFA induced glucagon secretion. We focused on the cellular expression, overexpression of GPR40, as well as antisense treatment and the intracellular and glucagon secretory responses related to these manipulations.

Materials and methods

Animals and islet isolation. Islets from female mice of the NMRI strain (B&K, Sollentuna, Sweden) weighing 25–30 g were used. The animals were given a standard pellet diet (B&K) and tap water ad libitum. For preparation of pancreatic islets mice were sacrificed by cervical dislocation and

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.193

the pancreata were filled by retrograde injection of a collagenase solution via the bile-pancreatic duct. Islets were then isolated and handpicked under a stereomicroscope and immediately used in the experiments. The experimental procedures were approved by the local Ethical Committee for Animal Research.

Reagents. Cy2-conjugated anti-rabbit IgG and Cy5-conjugated anti-guinea pig IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). Guinea pig-raised anti-glucagon antibody and the glucagon radioimmunoassay kit were from Euro-Diagnostica (Malmö, Sweden). Fatty acid free bovine serum albumin (BSA) was from Roche (Bromma, Sweden). All other chemicals were from Merck (Darmstadt, Germany) or Sigma (St. Louis, USA). Linoleic acid (LA) was dissolved in ethanol (95%) to a concentration of 100 mM. This stock solution was then diluted in culture or incubation media to the desired concentrations.

Confocal microscopy. Freshly isolated islets were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100 and unspecific sites were blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories). Mouse GPR40 was detected with a polyclonal antibody (1:100) in combination with Cy2-conjugated anti-rabbit IgG (1:100). The GPR40 antibody was raised in rabbit against the C-terminal peptide: NH_2-CVTRTQRGTIQK-COOH (Innovagen, Lund, Sweden). The specificity of this antibody was tested in a previous study [4]. For staining of glucagon, islets were incubated with a guinea pig-raised anti-glucagon (1:1000) antibody followed by incubation with a Cy5-conjugated anti-guinea pig IgG antibody (1:150). The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505–530 nm (Cy2) and 633/>650 nm (Cy5).

Cell lines. The hamster glucagonoma cell line, In-R1-G9, was kindly provided by Dr Jacques Philippe (University of Geneva, Geneva, Switzerland) and cultured in GlutaMAX-containing RPMI 1640 (11 mM glucose) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Pen-Strep). HEK293 cells were cultured in DMEM with GlutaMAX (25 mM glucose), with the addition of 10% FCS and Pen-Strep. All culture media and supplements were from Invitrogen (Paisley, UK) and the cells were cultured at 37 °C and 7% CO_2.

Receptor cloning. The hamster *gpr40* gene (Accession No. AB095746) was amplified from CHO cell cDNA in a PCR (forward primer, 5′-CA GGCGGCCGCCATGGCCCTGTCTCCCCAAC-3′; reverse primer, 5′-CAGGAATTCCTACTTCTGAATTGTTCCTCCTTGAGTC-3′) using the Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). After sequencing, the PCR product was ligated into the pIRESpuro vector (Clontech, Mountain View, CA) following digestion with *Not*I and *Eco*RI, creating the Ham40-pIRES vector.

Transfections. HEK 293 and In-R1-G9 cells were seeded in 6-well plates and transfected with the Ham40-pIRES vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The original pIRESpuro vector was used for sham transfections. For each transfection, 4 μg DNA per well was used and the transfection time was 6.5 h. The cells were cultured for another 48 h before being assayed to allow sufficient time for receptor expression.

Phosphatidyl inositol hydrolysis. GPR40 activation was assayed by the hydrolysis of phosphatidyl inositol (PI), as described [4,10]. Briefly, transfected cells were loaded with myo-[^{3}H]inositol overnight and stimulated with LA. The cells were lysed, the inositol phosphates (IPs) were extracted using anion exchange chromatography, and radioactivity was measured in a Beckman-Coulter LS6500 Scintillation Counter. To illustrate the change in PI hydrolysis, basal IP levels measured in native or sham-transfected cells were normalized to 1.

Glucagon release from In-R1-G9 cells. In-R1-G9 cells were seeded in 48-well plates. After reaching confluency, the cells were preincubated in 200 μl Krebs–Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mM Hepes, 1 mM glucose, and 0.1% (wt/vol) FA-free BSA (KRB) for 30 min in a 37 °C incubator. The preincubation medium was replaced by 500 μl KRB with test substances added at the indicated concentrations. To determine maximal secretory capacity, incubation with 10 mM L-arginine was included. After 60 min incubation, samples were immediately stored at –20 °C pending radioimmunochemical analysis for glucagon content. Special care was taken to ensure no cells were aspirated along with the medium.

Glucagon release from islets and antisense intervention. Freshly isolated pancreatic islets were preincubated for 30 min at 37 °C in KRB with 1% FA-free BSA. After preincubation the buffer was changed and the islets were incubated at 1.0 or 8.3 mmol/l glucose with or without LA for 60 min at 37 °C in an incubation box (30 cycles/min). Each incubation vial contained 12 islets in 1.0 ml of buffer solution and was gassed with 95% O_2 and 5% CO_2 to obtain constant pH and oxygenation. Aliquots of the media were removed immediately after incubation and frozen for the subsequent glucagon assay.

In the antisense experiments, the islets were first cultured for 48 h in RPMI 1640 with 10% FCS, 100 U/ml penicillin, 10 μg/ml streptomycin in the presence or absence of either a GPR40 specific or a nonsense morpholino oligonucleotide (Gene Tools, Philomath, OR) at a concentration of 1.4 μM. The morpholinos were loaded into the islets using the accompanying EPEI Special Delivery Solution according to the manufacturer's instructions. The islets were then thoroughly washed and subjected to incubation at 8.3 mmol/l glucose as described above.

Statistics. Statistical significance was assessed using unpaired Student's *t*-test and a *P* value of less than 0.05 was considered significant. Results are expressed as means ± SEM.

Results

Confocal microscopy

To investigate whether α-cells express GPR40, we performed a confocal microscopic study of freshly isolated mouse islets. Glucagon-containing cells were found in the periphery (Fig. 1a) and GPR40 was found abundantly expressed throughout the islet (Fig. 1b). The overlay (Fig. 1c) demonstrated coexpression of GPR40 and glucagon in a number of cells.

Transfection experiments

HEK293 cells were transiently transfected with the Ham40-pIRES vector. Sham-transfected HEK293 cells do not respond with PI hydrolysis after an LA challenge, but when expressing hamster-GPR40, stimulation with 100 μM LA resulted in a 4.8-fold increase in IP levels in these cells (data not shown), demonstrating that LA is an agonist also for the hamster homolog of GPR40. This con-

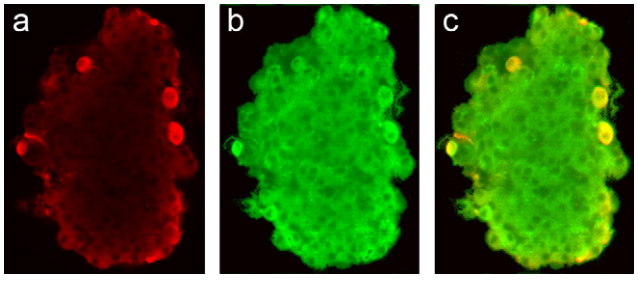

Fig. 1. Cross-staining of glucagon and GPR40 immunoreactivities. Confocal microscopy images of an isolated mouse islet, showing staining for glucagon (a), GPR40 (b), or both (c). The yellow color in the overlay (c) indicates expression of both glucagon and GPR40 in the same cells. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

clusion was confirmed in In-R1-G9 cells. Boosting the endogenous GPR40 expression in In-R1-G9 cells (readily detected in a standard real-time PCR with In-R1-G9 cDNA; forward primer 5′-GGCGTGTGTGTGGCTA TATG-3′, reverse primer 5′-ACGAAGAAGAGCAG GATGGA-3′) by transfection with Ham40-pIRES resulted in a tripling of the IP levels seen in the sham-transfected cells (Fig. 2). This was true for both the basal and the LA stimulated PI hydrolysis in the overexpressing cells.

LA stimulation of In-R1-G9 cells

In-R1-G9 cells were stimulated with increasing concentrations of LA and assayed for PI hydrolysis activity or glucagon release. At concentrations from 0 to 1 mM LA, the cells exhibited a dose-dependent increase in PI hydrolysis (Fig. 3a). The LA-induced rise in IP levels in the In-R1-G9 cells correlated with an increase in glucagon secretion when raising the LA concentration, at 1 mM glucose (Fig. 3b). In a control experiment, there were no significant differences between 10 mM L-arginine and 1 mM LA induced secretion (4570 ± 440 and 3980 ± 540 pg/well/h, respectively, $n = 8$ in each group).

Glucagon secretion from isolated islets

LA induced potentiation of glucagon release was studied in freshly isolated mouse islets at 1 and 8.3 mM glucose (Fig. 4). Basal glucagon secretion was higher at the lower glucose concentration, but under both conditions the addition of LA gave rise to a dose-dependently increased secretion at concentrations of 100 μM and above.

In the GPR40 knock-down experiments, the specificity of the response to LA was examined. The islets again displayed a dose-dependent glucagon secretion in response to LA stimulation at 8.3 mM glucose (Fig. 5a). At all LA concentrations, the responses were similar in the untreated

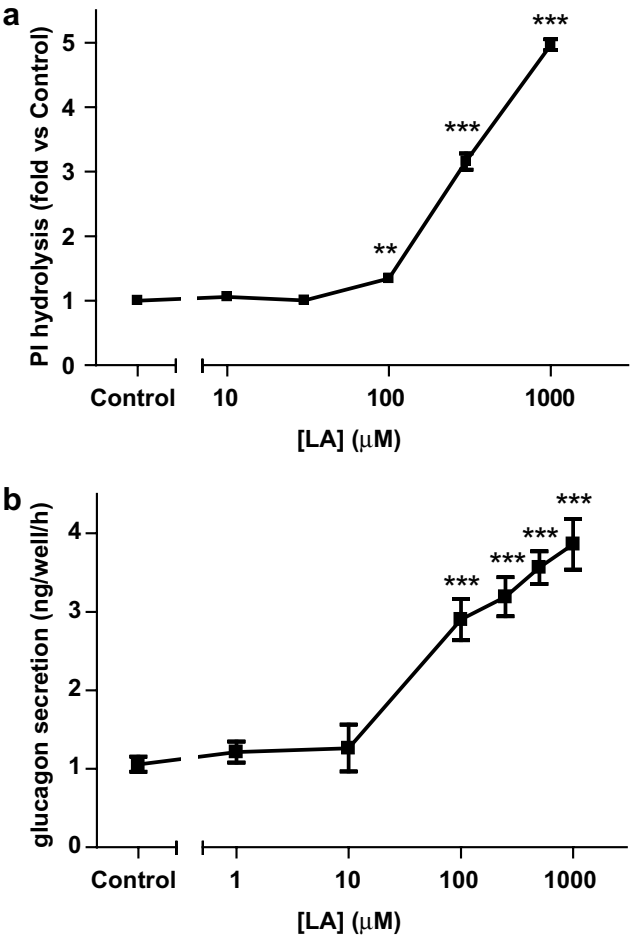

Fig. 3. In-R1-G9 cells are stimulated by LA. Stimulation of In-R1-G9 glucagonoma cells with LA gives rise to a dose-dependent response in both PI hydrolysis (a) and glucagon exocytosis (b). a: means from a representative experiment performed on two different occasions, $n = 3$ in each group; b: $n = 6$–14. $^{***}P < 0.001$, $^{**}P < 0.01$ (vs 0 μM LA).

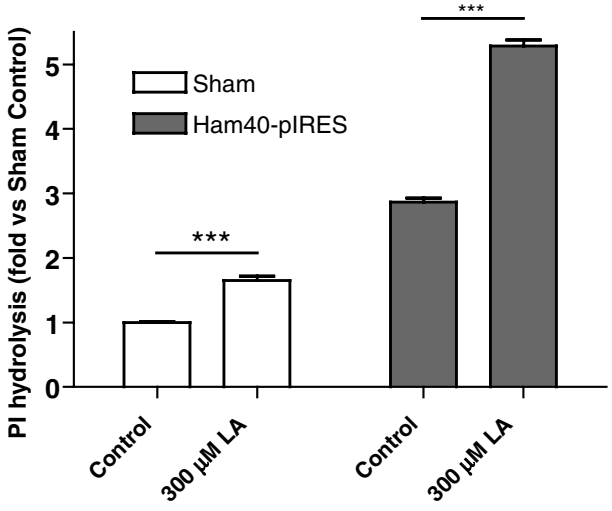

Fig. 2. PI hydrolysis in In-R1-G9 cells transfected with GPR40. Transfected cells exhibit increased levels of IPs after LA stimulation, compared to sham-transfected controls. Data are from a representative experiment performed on two different occasions, $n = 3$ in each group. $^{***}P < 0.001$.

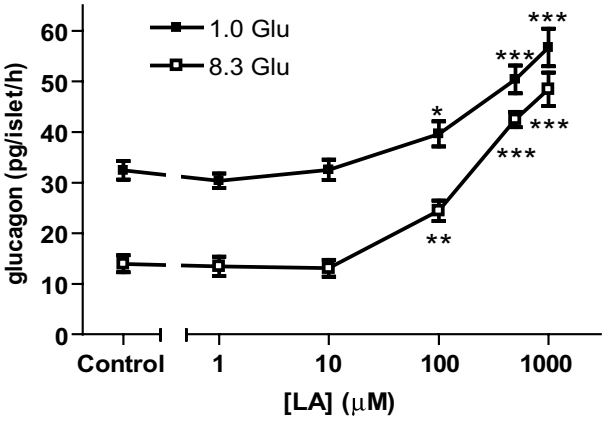

Fig. 4. LA stimulates glucagon secretion from freshly isolated mouse islets. At both 1 and 8.3 mM glucose, glucagon secretion from freshly isolated mouse islets is dose-dependently stimulated by LA, $n = 8$. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. 0 μM LA.

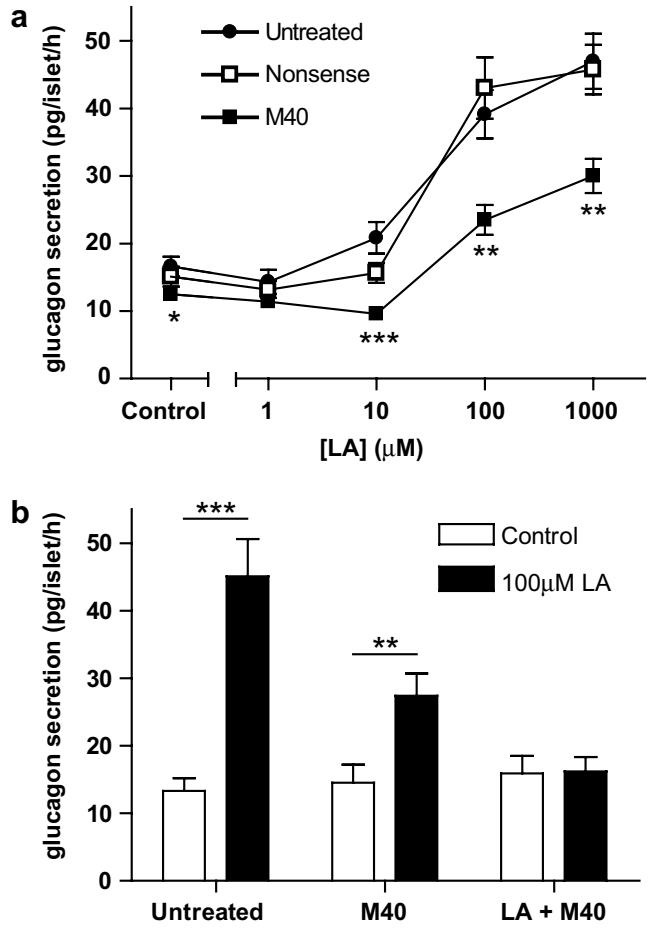

Fig. 5. Glucagon secretion from mouse islets stimulated with LA is dependent on GPR40. LA stimulation of glucagon release from isolated mouse islets is partially inhibited by the addition of the M40 antisense morpholino (a and b), but unaffected by a nonsense morpholino (a). This effect of LA is completely abolished by pretreatment of the islets with LA before applying the antisense (b). a: $n = 6$–8; b: $n = 8$. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ for M40 vs untreated (a) or for 100 μM LA vs 0 μM LA (b).

islets and in the islets treated with the nonsense morpholino, whereas glucagon secretion from the islets treated with the GPR40 specific morpholino, M40, was clearly reduced.

In a follow-up experiment, addressing potential residual GPR40 activity, the islets were pretreated with 100 μM LA for 30 min before application of the antisense morpholino for 48 h. The islets were then challenged with 100 μM LA for 1 h and assayed for glucagon release. Untreated islets exhibited a robust glucagon secretion that was markedly blunted by the M40 treatment (Fig. 5b). In the islets pretreated with LA prior to M40 treatment, the glucagon response due to LA stimulation was completely abolished.

Discussion

Although a number of early reports on the connection between FFAs and glucagon secretion indicated an inverse correlation [11,12], more recent observations have pointed towards the contrary, namely that FFAs instead exert a stimulatory effect on glucagon release from α-cells. Bollheimer et al. demonstrated that both acute palmitate and oleate stimulation resulted in increased glucagon secretion from isolated rat islets [7], and the same result was seen in mouse islets treated with palmitate [9]. A recent thorough investigation revealed that glucagon secretion from isolated mouse islets was enhanced by a spectrum of medium to long chain FFAs [8]. However, the mechanism behind the glucagonotropic actions of FFAs has still not been elucidated. Elevation of $[Ca^{2+}]_i$, via L-type Ca^{2+}-channels, has been implicated in the palmitate-induced release of glucagon [9], similar to what has been shown for GPR40-mediated insulin release from β-cells [6].

The primary goal of this investigation was to determine if the recently identified free fatty acid receptor, GPR40, which is abundantly expressed in pancreatic β-cells and when activated serves to augment glucose-stimulated insulin secretion, is expressed also in α-cells and whether it is involved also in fatty acid-induced glucagon release. Previous attempts to detect expression of GPR40 in α-cells have been unfruitful, both using quantitative PCR on one human glucagonoma [13] and in situ hybridization combined with immunostaining for islet hormones in the rat pancreas [1,2]. The overall islet contribution of the different islet cell types varies among species. Furthermore, the relative abundance of different cell types may also vary depending on the location of the islet within the pancreas, as demonstrated for α- and PP-cells. Islets from the proximal (head) part of the rat pancreas contain relatively few α-cells, but numerous PP-cells, whereas the relationship is reversed in the distal (tail) part [14,15]. Different isolation methods result in variable yields of islets and the origin of the islets will have a great influence on studies of e.g., α- and PP-cells. In the process of enzymatic islet isolation, care must also be taken not to disrupt cell surface proteins, especially when studying cells in the periphery of islets. In the present study, isolated mouse islets were double stained for GPR40, using an antibody employed in previous GPR40 expression studies [4], and for glucagon. The β-cells account for the majority of the cells in an islet, and therefore GPR40 staining is seen throughout the entire islet. In contrast, glucagon expressing α-cells are found predominantly in the periphery of mouse islets, and interestingly this is also where the double staining was detected, demonstrating an α-cellular expression of GPR40.

We found that the hamster glucagonoma cell line, In-R1-G9, expresses GPR40 endogenously. In order to use this cell line in our further experiments, we first determined that hamster GPR40 responds similarly to FFA stimulation as the more extensively studied mouse, rat, and human homologs. Untransfected HEK293 cells do not express GPR40 endogenously and are inert to LA stimulation when assayed for PI hydrolysis [1]. Transfection of HEK293 cells with the Ham40-pIRES vector, however, lead to a marked increase in phospholipid hydrolysis when challenging the cells with LA. This is in accordance with

our previous findings using the mouse receptor [4]. A gain-of-function was observed in In-R1-G9 cells overexpressing GPR40, further strengthening the hypothesis that a functional GPR40 is expressed in glucagon secreting cells and establishing this cell line as a suitable model system for studying its interaction with the receptor ligands. G-protein coupled receptors in a cell membrane oscillate between inactive and active conformations, where the periods in the active state contribute to the basal activity of the receptor [16]. As a consequence, a higher number of receptors expressed on the surface of a cell leads to a higher overall basal activity, which is also illustrated by our data. The gain-of-function (an increased PI hydrolysis) in the overexpressing cells lends support to LA stimulated IP generation in native In-R1-G9 cells being mediated by GPR40.

Using monocultures it is possible to examine the direct effect of FFAs on the α-cell, without the potential paracrine influence of other islet hormones released in response to the FFAs. The In-R1-G9 cells do not secrete detectable concentrations of other islet hormones [17], but exposed to different concentrations of LA, In-R1-G9 cells dose-dependently secrete glucagon. It is noteworthy that the effect of LA on glucagon secretion was paralleled by an increased PI hydrolysis, an established event in the downstream signaling of GPR40, and that although the increase in IP production is only minor (but statistically significant) at 100 μM LA, the effect on glucagon secretion is substantial.

In the last part of the study, we examined glucagon secretion from isolated mouse islets. Confirming our results from the clonal cells, subjecting freshly isolated islets to LA we found a dose-dependency in the glucagon secretory response. As expected, increasing the glucose concentration from 1 to 8.3 mM resulted in a decreased basal glucagon secretion. At both concentrations, however, the addition of LA caused a dose-dependent increase of the amount of glucagon released from the islets. The increase in secretion was more robust at the higher glucose concentration, possibly because much of the glucagon exocytotic machinery is already employed at low glucose levels. The additive nature of LA on glucagon secretion supports a situation similar to that in β-cells, where activation of GPR40 augments insulin release after challenge with its agonist FFAs, e.g., LA [2,4–6].

To further pin-point the importance of GPR40 stimulation in α-cell function, glucagon secretion from isolated islets was assayed after GPR40 antisense intervention. The addition of the sequence specific antisense morpholino, M40, resulted in a reduced glucagon secretion in response to LA stimulation, identical to the described reduction in FFA-potentiated insulin secretion [4]. Unspecific effects caused by morpholino chemistry can be ruled out since glucagon secretion in islets subjected to a nonsense morpholino was unaffected. Morpholinos prevent the translation of targeted mRNA by sterically blocking the translation initiation complex [18] and therefore the M40 is unable to modulate the GPR40 receptors already expressed on the surface of the islet cells. This could be a reason behind the partiality of the response knock-down effect. To address this, we pretreated the islets with LA before the application of the antisense oligos. Receptor stimulation often leads to internalization of the receptors, after which they are either recycled to the surface or broken down and have to be replenished by de novo synthesis [19]. The combined LA and M40 treatment of the islets effectively abolished the augmentation of glucagon secretion seen in untreated cells in response to LA-stimulation. Exposure of islets to palmitate has been shown to dose-dependently lower intracellular glucagon content in vitro [7] and a preceding agonist-induced receptor challenge can also affect a subsequent restimulation, but neither of those mechanisms are likely to play a role in our experiments, as our LA pretreatment only lasted for 30 min and was then followed by a 48 h incubation before measuring glucagon release in response to LA restimulation.

With its established actions in β-cells, the inhibition of GPR40 activity has been postulated to be beneficial to individuals with elevated levels of plasma FFAs [2]. Indeed, β-cellular ablation of GPR40 expression in mouse has been shown to be protective against many of the aspects of the metabolic syndrome often following in the foot steps of obesity [5]. The implication of GPR40 in the regulation of glucagon release makes it conceivable that antagonising GPR40 would have the added beneficial effect of lowering hepatic glucose output, thereby alleviating not only the strain put on the β-cells by a chronically high concentration of FFAs, but also the stress induced by hyperglycaemia in an often increasingly insulin-resistant body. In fact, suppression of glucagon secretion was discussed as a potential intervention in diabetes already 30 years ago [20]. This further underlines the potential therapeutic relevance of this important nutrient sensing receptor.

Is summary, we demonstrate for the first time, expression of the FFA receptor, GPR40, in pancreatic α-cells. This is reflected functionally by a dose-dependent response to LA stimulation in the glucagonoma cell line, In-R1-G9, as assayed by both the generation of intracellular second messenger IPs and by glucagon secretion. Overexpression of GPR40 further increases the In-R1-G9 cell responsiveness to LA. Isolated mouse islets respond to LA exposure by secreting glucagon and antisense experiments show that this response is, at least in part, dependent on the activation and function of GPR40.

Acknowledgment

This work was in part supported by GS Development, Malmö, Sweden.

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