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The relationship between GPR40 and lipotoxicity of the pancreatic β-cells as well as the effect of pioglitazone

Peiwen Wu, Liyong Yang *, Ximei Shen

Department of Endocrinology, The First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian 350005, China

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ABSTRACT

Free fatty acids (FFAs) acutely stimulate insulin secretion from pancreatic β-cells, whereas impair β-cell function following long term exposure. GPR40, a FFAs receptor, has been demonstrated to be activated by both medium and long chain FFAs and played an important role in insulin release. This study was performed to determine the contribution of GPR40 to short- and/or long-term effects of FFAs on glucose-stimulated insulin secretion (GSIS) and the expression of PDX-1 and GLUT2 in pancreatic β-cells, as well as the intervenient effects of pioglitazone on lipotoxicity of β-cells. βTC6 cell line stably expressing GPR40shRNA were established and the intervention of FFAs and pioglitazone on GSIS and expression of PDX-1 and GLUT2 in βTC6 cells was investigated. Results showed that 1-h exposure to FFAs significantly enhanced GSIS and increased expression of PDX-1 and GLUT2 in pSilencer-control transfected cells, but not in cells transfected with GPR40shRNA. While 48-h exposure to FFAs significantly impaired GSIS in pSilencer-control transfected cells as well as cells transfected with GPR40shRNA. Furthermore, pioglitazone enhanced insulin secretion in pSilencer-control transfected cells exposed to FFAs for 48 h, but not in cells transfected with GPR40shRNA. These results indicate that GPR40 mediates the short-term effects of FFAs on GSIS, but does not mediate the chronic lipotoxicity on β-cells. The reverse role of pioglitazone on lipotoxicity of β-cells may be related to GPR40.

1. Introduction

Free fatty acids (FFAs) are recognized to influence insulin secretion of pancreatic β-cells. It has been demonstrated that under physiological conditions, FFAs acutely improves insulin secretion from pancreatic β-cells, whereas results in β-cell dysfunction following chronically exposure [1]. The FFAs receptor, G-protein-coupled receptor 40 (GPR40), is a member of large family highly expressed in rodent and human pancreatic insulin-producing β-cells [2–4] and has been proposed to mediate insulin secretion, hence to play an important pathophysiological role in type 2 diabetes mellitus [1,5–9].

Despite intensive research efforts, the physiological role of GPR40 still remains unclear. In order to gain a better understanding for GPR40, this study was conducted to investigate the role GPR40 plays in pancreatic islet cells, including the effect of GPR40 on both short and long-term FFAs on GSIS, the relationship between GPR40 and FFAs mediated expression of a number of key islet proteins, such as PDX-1 and GLUT2 as well as the GPR40 contribution to the thiazolidinedione reverse role on lipotoxicity of β-cells.

2. Materials and methods

2.1. βTC6 cell culture

The mouse insulinoma cell line βTC6 was obtained from the American Type Culture Collection and grown at 37 °C under a humidified, 5% CO_2/95% air (v/v) atmosphere in Low Glucose (5.6 mM) DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were subcultured every week. Cells from passage 30 to 70 were used for all experiments.

2.2. GPR40 siRNA duplexes and plasmid constructs

siRNA was designed to interfere with the expression of mouse GPR40 mRNA, referring to technical information (Ambion, Austin, TX). The sequences of the two 21-nucleotide GPR40 siRNA duplexes were:

duplex 1, AACATACCCGTGAATGGCTCC;
duplex 2, AATATGTGTGACGAGGACTCA.

The target sequence of the GPR40 siRNA duplexs was then used to design two complementary 55-mer siRNA template oligonucle-

* Corresponding author. Fax: +86 059183318716.
E-mail address: liyongy@yahoo.com (L. Yang).

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otides encoding GPR40 short hairpin RNAs (shRNAs) with BamH1 and HindIII overhangs. The two oligonucleotide used were:

GPR40 shRNA1: 5′-GATCC CATACCCGTGAATGGCTCC TTCAAGA GA GGAGCCATTCACGGGTATGTT A-3′ and 5′-AGCTT AACATAC CCGTGAATGGCTCC TCTCTTGAA GGAGCCATTCACGGGTATG G-3′; and GPR40 shRNA2: 5′-GATCC TATGTGTGACGAGGACTCA TTCA AGAGA TGAGTCCTCGTCACACATATT A-3′ and 5′-AGCTT AATAT GTGTGACGAGGACTCA TCTCTTGAA TGAGTCCTCGTCACACATA G-3′.

Forward and reverse oligonucleotides were incubated in DNA annealing solution for 3 min at 90 °C, followed by incubation for 1 h at 37 °C. The annealed DNA for siRNA was ligated with linearized pSilencer 4.1-CMV neo siRNA expression vector (Ambion) at BamHI and HindIII sites. After transformation, the sequence was further identified by sequencing from both sides. Then, the identified recombinant plasmid constructs were prepared with Qiagen Endofree Plasmid kits. The vector, pSilencer 4.1-CMV neo negative control (Ambion), was used as the negative control plasmid in all experiments.

2.3. Transfection

βTC6 cells were seeded in 6-well plates (5.0×10^5 cells/well). On the following day, when cells reached about 70–80% confluence, they were transfected with 4 μg above mentioned recombinant plasmid using 10 μl Lipofectamine2000 (Invitrogen) as described by the manufacturer. Negative control cells were transfected with the negative control plasmid. One day after the transfection, G418 (800 μg/ml) was added. Resistant clones were picked after 2 weeks and analyzed for expression of GPR40 by Western blot.

2.4. Protein extraction and Western blot

Cells were lysed with ice cold radioimmune precipitation assay (RIPA) buffer and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) on ice for 20 min. Lysates were centrifuged at 12,000*g* for 10 min, and the supernatant containing the cellular protein was collected. Equal amount (25–50 μg per sample) total protein of each sample was boiled, electrophoresed on SDS–PAGE using 10% separating gels, and transferred to polyvinylidene fluoride (PVDF) (Invitrogen). Primary antibodies were used at the following working dilutions: rabbit anti-human GPR40 polyclonal antibody (1:200 dilution, sc-32905, Santa Cruz Biotechnology); goat anti-human PDX-1 polyclonal (1:200 dilution, sc-14664, Santa Cruz Biotechnology); rabbit anti-human GLUT2 polyclonal antibody (1:200 dilution, sc-9117, Santa Cruz Biotechnology); mouse monoclonal antibody β-actin (1:1000 dilution, sc-47778, Santa Cruz Biotechnology). For β-actin, the blot was incubated with alkaline phosphatase-labeled anti-mouse IgG antibody (1:2000 dilution, Santa Cruz Biotechnology). For GPR40, PDX-1 and GLUT2, the alkaline phosphatase-conjugated secondary antibody and ECL system provided by the WesternBreeze kit (Invitrogen) was used according to the manufacturer's instructions.

2.5. FFAs and pioglitazone intervention

Cells were incubated for 24 h to attach before changing the medium. Cells were then incubated in 0.5 mmol/L FFAs (a 2:1 mixture of oleate: palmitate, Sigma) for 1 h or 48 h, or 10 μmol/l pioglitazone (Jiangsu Hengrui Medicine Co., Ltd., China) or combination of FFAs and pioglitazone for 48 h. FFAs were dissolved in ethanol and the final concentration of BSA was adjusted to 0.5% (w/v). Control medium was carried out in the presence of BSA/ethanol in DMEM without FFAs. Pioglitazone was dissolved in DMSO and the final concentration of DMSO was adjusted to 0.1% (v/v); the medium containing the same amount of DMSO was used as the control.

2.6. Glucose-stimulated insulin assay

Cells were plated at a density of 40,000 (cell/well) in 96 well plates. After treated with FFAs or pioglitazone or control medium, cells were washed twice with glucose-free Krebs–Ringer bicarbonate HEPES buffer (KRBH) (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 25 mM HEPES, pH 7.4) and pre-incubated at 37 °C for 40 min with the glucose-free Krebs/HEPES Ringer solution. Cells were then washed once with glucose-free KRBH and incubated for 60 min at 37 °C in KRBH containing either 0.5 or 2.8 mmol/L glucose. Aliquots of supernatant were collected and stored at −20 °C for insulin determination by radioimmunoassay kit (Beijing North Institute of Biological Technology).

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test for unpaired data when two samples were compared. Data were expressed as means ± SEM. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. The recombinant plasmid knocked down GPR40 protein in βTC6 cells

βTC6 cells transfected with GPR40shRNA caused a significant decrease in the expression of GPR40 when compared to negative control (NC) shRNA transfected cells. Relative to β-actin, the band intensity of GPR40 decreased remarkably by about 71% in the GPR40shRNA11. Transfection of βTC6 cells with NC shRNA had no significant effect on the expression of GPR40 compared to transfection reagent exposed βTC6 cells (BC) (Fig. 1).

3.2. Acute effects of FFAs on GSIS in GPR40shRNA transfected βTC6 cells

The role of GPR40 for short-term FFAs-stimulated insulin secretion was studied in βTC6 cells (Fig. 2). A prior report with βTC6 cells indicated that the maximal release of insulin occurred at approximately 3 mM glucose [10]. We observed that the peak value of glucose- stimulated insulin secretion in βTC6 cells appeared under 2.8 mM glucose stimulation. In βTC6 cells transfected with GPR40shRNA, GSIS was similar to NC or BC. 1-h exposure to 0.5 mmol/L FFAs significantly enhanced GSIS in NC or BC ($P < 0.01$), but not in cells transfected with GPR40shRNA ($P > 0.05$).

3.3. Chronic effects of FFAs and pioglitazone on GSIS in GPR40shRNA transfected βTC6 cells

To investigate whether GPR40 mediated the long-term effect of FFAs on GSIS, cells were exposed to FFAs for 48 h. Results demonstrated that the 48-h exposure to FFAs equally and significantly impaired GSIS in BC, NC transfected cells as well as cells transfected with GPR40shRNA. Then the following experiment was conducted to study whether pioglitazone interacts with lipotoxicity of β-cells via GPR40. 48-h exposure to pioglitazone did not have any effect on GSIS in BC, NC and GPR40 shRNA transfected βTC6 cells cultured in absence of FFAs. Furthermore, pioglitazone enhanced insulin secretion in BC, NC transfected cells during culture with FFAs for 48 h, but not in cells transfected with GPR40shRNA (Fig. 3).

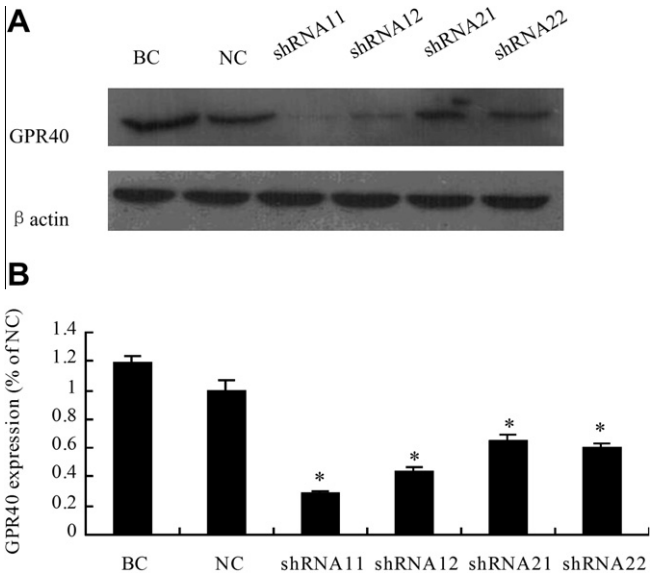

Fig. 1. GPR40 expression analysis in βTC6 cells. (A) Western blot experiments illustrating GPR40 protein expression in βTC6 cells transfected with GPR40shRNA (shRNA11, shRNA12, shRNA21, shRNA22), with the negative control plasmid (NC), and transfection reagent exposed βTC6 cells (BC). β-actin was used as controls. (B) Quantification of GPR40 protein expression levels in βTC6 cells. Data are presented as % of GPR40 expression in NC shRNA-transfected cells. The data represent means ± SEM of three independent experiments. *$P < 0.05$.

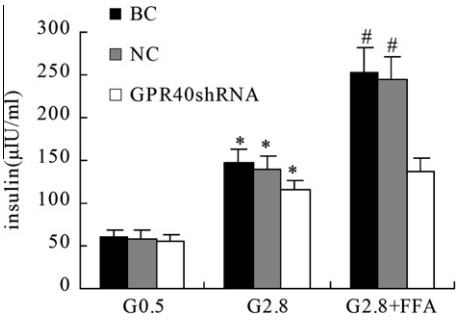

Fig. 2. Acute effects of FFAs on GSIS in βTC6 cells. The effect on GSIS after 1 h exposure to 0.5 mmol/L FFAs (a 2:1 mixture of oleate:palmitate) was measured using BC,NC and GPR40 shRNA transfected βTC6 cells. The cells were stimulated by 0.5 or 2.8 mM glucose. Data represent means ± SEM of three to five independent experiments. (*) Significant effect to G0.5; (#) significant effect to G2.8; (*, #) $p < 0.05$.

3.4. Effects of FFAs on the expression of PDX-1 and GLUT2 in GPR40shRNA transfected βTC6 cells

To determine whether effects of FFAs on changes of PDX-1 and GLUT2 in βTC6 cells were associated with GPR40, PDX-1 and GLUT2 expression was analyzed by Western blot in GPR40shRNA transfected βTC6 cells. Acute treatment (1 h exposure) of the cells with FFAs increased PDX-1 and GLUT2 expression in NC transfected cells, but not in cells transfected with GPR40shRNA, whereas chronic treatment with FFAs (48 h exposure) reduced PDX-1 and GLUT2 expression in NC transfected cells as well as cells transfected with GPR40shRNA (Fig. 4).

4. Discussion

It is now clear that FFAs exert pleiotropic effects on pancreatic β-cells. FFAs improves insulin secretion in the short term incubation with pancreatic β-cells [11–13], however chronic exposure impairs β-cell function [12,14–16] and leads to β-cell apoptosis [16–18]. A number of *in vitro* and *in vivo* studies have now demonstrated that acute treatment with FFAs on β-cells promotes insulin release via GPR40 [6,7]. FFA-stimulated Ca^{2+} signal and insulin secretion through the activation of GPR40 in pancreatic β-cells [19]. Our data indicated that FFAs lost part of their actions on GSIS in cells transfected with GPR40shRNA, thus demonstrating that GPR40 mediate, at least in part, the acute stimulatory action of FFAs on GSIS.

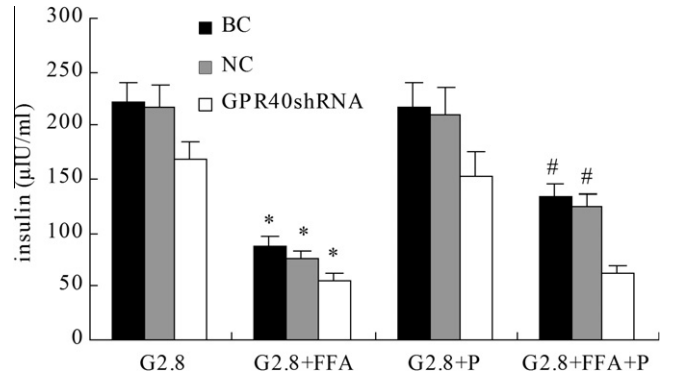

Fig. 3. Chronic effects of FFAs and pioglitazone on GSIS in βTC6 cells. The effect on GSIS after 48 h exposure to 0.5 mmol/L FFAs or 10 μmol/l pioglitazone or mixture of both was measured in BC, NC and GPR40 shRNA transfected βTC6 cells. The cells were stimulated by 2.8 mM glucose. Data represent means ± SEM of three to five independent experiments. (*) Significant effect to G2.8; (#) significant effect to G2.8 + FFA; (*, #) $p < 0.05$.

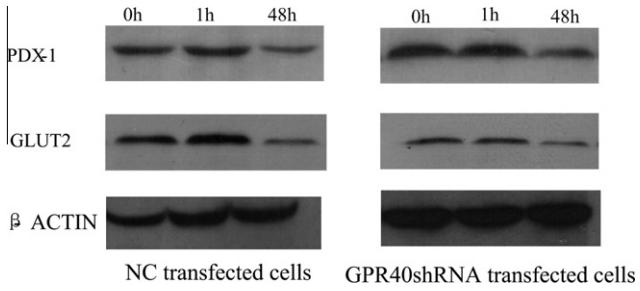

Fig. 4. Effects of FFAs on the expression of PDX-1 and GLUT2 in βTC6 cells detecting by Western blotting. NC transfected cells and GPR40shRNA transfected cells were stimulated with 0.5 mmol/L FFAs for 0, 1 h and 48 h. Western blotting was performed using anti-PDX-1, anti-GLUT2, and anti-β-actin antibodies. Representative Western blotting analysis is shown.

There is a matter of debate on whether GPR40 mediates the chronic inhibitory effects of FFAs on GSIS. Steneberg et al. showed that insulin promoter factor 1 – GPR40 transgenic mice were glucose-intolerant and hypoinsulinemic when fed a normal diet. In contrast, GPR40 knockout mice, when fed a high-fat diet, did not develop glucose intolerance [5]. This finding indicated that GPR40 may mediate both the short- and long-term effects of fatty acids on β-cell function. On the other hand, studies using GPR40 knockout mice showed that deletion of GPR40 did not protect against the effects of long-term fatty acid exposure [6–9]. Islets from the GPR40 knockout mice appear to be as vulnerable as wild-type islets to the detrimental effects from FFAs *in vitro*. The results presented in this study demonstrated that GPR40 only mediate short-term, but not long-term effects of FFAs on GSIS in βTC6 cells. It has been confirmed that 48-h exposure to FFAs caused comparable inhibition of GSIS in pSilencer – control transfected cells as well as cells transfected with GPR40shRNA.

The transcription factor PDX-1 is abundantly expressed in β-cells and regulates the expression of genes to ensure proper GSIS and β-cell function [20–22]. GLUT2 is one of the genes that regulated by PDX-1. Recently, data showed that PDX-1 bind to an enhancer element within the 5′-flanking region of GPR40 [23], and it was additionally found that loss of Ipf1/Pdx-1 in β-cells impairs GPR40 expression in Ipf1/Pdx1-null mice. It hence indicated that PDX-1 is required for GPR40 expression in β-cells. It has been shown by previous studies that the exposure of β-cells into FFAs could affect the level of PDX-1 and GLUT2 expression, further research was conducted in this study to investigate the relationship between GPR40 and FFA affected PDX-1 and GLUT2 expression. In this study, GPR40shRNA βTC6 cells showed decreased expression of both PDX-1 and GLUT2 in response to acute exposure to FFAs with a concomitant reduction in GSIS .These findings indicated the role of GPR40 in FFAs stimulation of PDX-1 and GLUT2 expression and insulin secretion.

Thiazolidinediones are traditionally described as peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists that act to reverse insulin resistance in target tissues, while not all of the effects of thiazolidinediones can be explained by their action on PPAR-γ. It has recently been identified as a partial agonist at endogenously expressed GPR40 [24]. The results in the present study showed that pioglitazone enhanced insulin secretion in pSilencer – control transfected cells treated with FFAs for 48 h, but not in cells transfected with GPR40shRNA.This favor the view that the deleterious action of FFAs on the β-cells is counteracted by the thiazolidinedione drug partly through GPR40.

In summary, we have presented *in vitro* evidence showing that GPR40 mediate the short-term effects of FFAs on GSIS through the role of PDX-1 and GLUT2. GPR40 does not mediate the chronic toxic effects of FFAs on β-cell function. The reverse role of pioglitazone on lipotoxicity of β-cells may be related with GPR40.

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