The role of G protein-coupled receptor 40 in lipoapoptosis in mouse β-cell line NIT-1

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Abstract

Free fatty acids (FFAs) exert divergent effects on β-cells. Acute exposure to FFAs stimulates insulin secretion, whereas chronic exposure impairs β-cell function and induces apoptosis. The G protein-coupled receptor 40 (GPR40) is preferentially expressed in β-cells and is activated by a wide range of FFAs. In this study, we used small interfering RNA technology and apoptosis assay in mouse β-cell NIT-1 to address the role of GPR40 in β-cell lipoapoptosis and function. Results showed that palmitate induced β-cell apoptosis, which was not mediated through GPR40, whereas oleate protected NIT-1 cells from palmitate-induced lipoapoptosis, which was mediated at least in part through GPR40. Moreover, by detecting the activation of the phosphatidylinositol 3-kinase and MAP kinase (MAPK) pathways, we found that oleate promoted the activation of extracellular signal-regulated protein kinase–MAPK pathway mainly via GPR40, increased the expression of early growth response gene-1, leading to the anti-lipoapoptotic effect on NIT-1 cells. It was suggested that GPR40 might be implicated in the control of β-cell mass plasticity and GPR40 probably provide a link between obesity and type 2 diabetes.

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Introduction

Type 2 diabetes (T2D) is tightly linked to obesity, which is characterized by hyperlipidemia and elevated circulating free fatty acids (FFAs; Kashyap et al. 2003, Moller & Kaufman 2005). Long-chain FFAs have important effects on pancreatic β-cell function. Acute treatment augments glucose stimulated insulin secretion (GSIS; Zraika et al. 2002, Haber et al. 2003, Yane & Corkey 2003); chronically elevated FFA levels interfere with β-cell function by induction of secretory failure (lipotoxicity; Lee et al. 1994, Zhou & Grill 1995, Lupi et al. 2002, Haber et al. 2003) and β-cell apoptosis (lipoapoptosis; Shimabukuro et al. 1998a,b, Lupi et al. 2002a,b). FFAs-induced apoptosis of pancreatic β-cells has been regarded as a critical determinant in switching from obesity and insulin resistance to T2D (Shimabukuro et al. 1998a,b, Unger & Zhou 2001). Whereas, in most instances, the degree of saturation of the fatty acids seems to be important for the cytotoxic effect, because saturated FFAs cause marked apoptosis (Maedler et al. 2001, 2003, El-Assaad et al. 2003) and unsaturated FFAs are much less cytotoxic. Furthermore, unsaturated FFAs have been shown to protect against the proapoptotic effects of saturated fatty acids (Eitel et al. 2002, Maedler et al. 2003). The mechanism underlying these actions is not well understood.

G protein-coupled receptor 40 (GPR40), which is a member of GPRs, has been shown to be preferentially expressed in pancreatic β-cells and in insulin-secreting β-cell lines (Briscoc et al. 2003, Itoh et al. 2003, Kotarsky et al. 2003, Itoh & Hinuma 2005, Tomita et al. 2005, 2006), it functions as a receptor for a wide range of saturated FFAs from C12 to C18 and unsaturated FFAs from C18 to C22 (Briscoc et al. 2003, Itoh et al. 2003, Kotarsky et al. 2005). Recent studies documented that GPR40 mediates both acute stimulatory and chronic inhibitory effects of FFAs on insulin secretion and that GPR40 signaling is linked to impaired glucose homeostasis (Itoh et al. 2003, Itoh & Hinuma 2005, Shapiro et al. 2005, Steneberg et al. 2005, Tomita et al. 2006). However, other physiological functions of GPR40 triggered by FFA stimulation are uncertain. It remains to be identified whether GPR40 is implicated to mediating lipoapoptosis of pancreatic β-cells.

The aim of this study is to determine whether GPR40 is implicated in FFA-mediated lipoapoptosis in NIT-1 cells. We silenced the expression of GPR40 by using small interfering RNA (siRNA) approach and tested the cell apoptosis and function in response to saturated and unsaturated fatty acid treatments in NIT-1 cells.

Materials and methods

Cell culture and treatment

The insulinoma cell line NIT-1 deriving from nonobese diabetic/large T-antigen (NOD/LT) mice (Hamaguchi et al. 2005, 2006) was used in this study. NIT-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic (AB-AMK; Gibco). NIT-1 cells were passaged using 0.25% trypsin-EDTA (Invitrogen) and cultured at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.
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et al. 1991) was kindly gifted by Prof. Yiming MU (General Hospital of PLA, Beijing, People’s Republic of China). NIT-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; Hyclone) and 1% penicillin–streptomycin. The medium was incubated at 37 °C in a humidified atmosphere of 5% CO2. The medium was refreshed every three days. NIT-1 cells of passages 20–40 in actively growing condition were used for the experiment. To assess the effects of FFAs (Sigma) on NIT-1 cell apoptosis, each BSA-bound FFA was dissolved in ethanol and the final concentration of BSA was adjusted to 0-5% (w/v). Control medium was carried in ethanol and the final concentration of BSA was 8 mmol/L. The medium was incubated at 37 °C in a humidified atmosphere of 5% CO2. To assess the effects of FFAs (Sigma) on NIT-1 cell apoptosis, each BSA-bound FFA was 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 FFA. To assess the effects of PD98059, nimodipine, and U73122 (Sigma) on NIT-1 cell apoptosis, PD98059 (50 μM), nimodipine (10 μM), or U73122 (3 μM) was dissolved in DMSO respectively; 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.

siRNA preparation and transfection

Three siRNAs were designed from the mouse GPR40 cDNA sequence (EMBL/GenBank/DDJ accession no. AF539809). These siRNAs, containing 21 nucleotides, were synthesized by Ambion, Austin, TX, USA. The sense sequences targeted by GPR40 siRNAs were 5'-GUGUGGUACUAACCCACUtt-3' (GPR40 siRNA1), 5'-ACAUACCUGUAAUGCUUtt-3' (GPR40 siRNA2), and 5'-CGAGGACUAAAGAGGAAUtt-3' (GPR40 siRNA3). Selected siRNA target sequences were also submitted to Basic local alignment search tool (BLAST) sequence against the mouse genome sequence to ensure that only the GPR40 gene targeted. The Scramble siRNA duplex (Ambion) was used as non-specific siRNA control. NIT-1 cells were plated at 50–70% confluence and transfected with siRNA complexes or only with transfection reagents (mock transfection) using siPORT NeoFX transfection reagent (Ambion) according to the manufacturer’s instructions. The concentration of siRNA for transfection was 50 nM. The cells were washed after 12-h transfection and resuspended in complete growth medium with serum for further experiments.

RT-PCR for GPR40

After 24, 48, and 72 h of transfection, total RNA was isolated from mock- and siRNA-transfected cells using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration and quality were assessed spectrophotometrically at wavelengths 260 and 280 nm. RT reaction was performed with random hexamer primers (Gene Link, Hawthorne, NY, USA) and an Omniscript RT kit (Qiagen). Equal amount of RNA (200 ng) was used as templates in each reaction. Primer sequences for GPR40 were 5'-cttttggccctggctac-3' (forward) and 5'-cctgtgatgatgccac-3' (reverse); for β-actin, the primers were 5'-gccttttgagctgatt-3' (forward) and 5'-ctggggcctgcaatta-3' (reverse). The cycling conditions for GPR40 were as follows: initial denaturation at 94 °C for 2 min followed by 25 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min. The PCR products were separated on 1-5% agarose gel. For the semi-quantification, an image of gel was captured, and the intensity of the bands was quantitated using the AlphaImager gel analysis system. The relative expression level of GPR40 was normalized to that of β-actin.

Western blotting

The cells were lysed with RIPA buffer, lysates were centrifuged for 20 min at 14 000 g, and protein was quantified using the Bradford method (Bio-Rad). Equal amounts (25–50 μg per sample) of protein extracts were then separated by 12% SDS-PAGE under reducing conditions and electrotransferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked in 5% (w/v) skimmed milk dissolved in 1×TBS-T buffer (14 mM Tris, 154 mM NaCl, and 0.05% Tween 20, pH 7.5) overnight at 4 °C. Primary antibodies are incubated for 1 h at room temperature in 5% (w/v) BSA dissolved in 1×TBS-T. Primary antibodies were used at the following working dilutions: GPR40 (1:500 dilution, sc-28416, Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (1:1000 dilution, sc-47778, Santa Cruz Biotechnology); and early growth response gene-1 (Egr-1; 1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA). Appropriate secondary antibodies conjugated to horseradish peroxidase (sc-2020; sc-2005; sc-2004, Santa Cruz Biotechnology) were incubated with respective membranes for 1 h at room temperature. Following five times of intermittent washes with 1×TBS-T, the membranes were processed for autoradiography using ECL test kit (Cell Signaling Technology). The results were quantified by densitometric analysis using the Image-Quant software.

Apoptosis assay

Following a 2-h preincubation under serum-free conditions, the cells were kept for 48 h in DMEM containing FFA at the indicated concentrations. Then, cell apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling.
(TUNEL) method and flow cytometry analysis (annexin V/PI staining) respectively. For TUNEL assay, 48 h after incubation with FFA, the cells were harvested, washed with PBS, fixed, permeabilized, and subjected to TUNEL labeling using an in situ cell death detection kit (Roche) according to the manufacturer’s protocol. The percentage of apoptosis was calculated as the percentage of cells’ definite positive TUNEL staining and was obtained by counting five randomly chosen fields in each slide under microscopic field of 250X. All TUNEL assays were read by the same investigator who was blinded to the treatment of the cells. For flow cytometry analysis, 48 h after incubation with FFA, 5×10⁵ cells/well onto six-well plates were harvested, washed with PBS, and then subjected to annexin V/PI staining using an AnnexinV/FITC Kit (Roche). Percentages of apoptotic cells were detected as the annexinV (+)/PI (−) fraction in the flow cytometer and analyzed with CellQuest software.

Measurement of the kinase phosphorylation

To detect the activation of Akt/protein kinase B (Akt), c-jun NH2-terminal kinase (JNK), ERK, and p38 MAP kinases (p38 MAPK) in NIT-1 cells, cells were incubated with oleate or palmitate (500 μM for each) for varying time intervals after a 12-h period of serum starvation, then total cell extracts were prepared, and subjected to western blotting as mentioned above. Western blotting using anti-phospho- and anti-total kinase antibodies (Cell Signaling Technology) was performed with 30 μg lysates. Membranes were first probed with a specific anti-phosphorylated antibody, then stripped and probed with the corresponding specific antibody against total protein. Results obtained with anti-phosphorylated antibodies are expressed as a percentage of the corresponding full protein.

Insulin secretion measurements

NIT-1 cells were plated in six-well plates at a density of 3×10⁵ cells/well, and siRNA transfection was performed as described above. On day 3 after the transfection, the cells were washed twice with glucose-free Krebs–Ringer bicarbonate Hepses buffer (KRBH) containing 140 mM NaCl, 3-6 mM KCl, 0-5 mM NaH₂PO₄, 0-5 mM MgSO₄, 1-5 mM CaCl₂, 2 mM NaHCO₃, 10 mM Heps, pH 7-4, and 0-01% BSA (fatty acid-free) and incubated at 37°C for 30 min in glucose-free KRBH. Cells were then washed once with glucose-free KRBH and incubated for 60 min at 37°C in KRBH containing the indicated stimuli. After 60 min, supernatant from each plate was collected and stored at −20°C until assayed for insulin. Insulin was measured by an ELISA kit (Linco, St Charlos, MO, USA) according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was carried out by using either Student’s t-test or ANOVA test for comparison of more than two variables. The data are presented as means±s.e.m. of at least three independent experiments. A P value <0.05 was considered statistically significant.

Results

Expression of GPR40 after treatment with GPR40 siRNA

In this study, to test whether siRNA could modulate GPR40 expression in mouse insulinoma NIT-1 cells, we treated NIT-1 cells with three GPR40 siRNAs respectively. GPR40 expression was analyzed by RT-PCR and western blotting. RT-PCR experiment, carried out in NIT-1 cells collected at different intervals after a 12-h transfection with 50 nmol/l of each GPR40-specific siRNA, showed a reduction of GPR40 mRNA when compared with transfection reagent-exposed cells (mock; Fig. 1A). The percentages of inhibition of GPR40 mRNA were 72-3% (GPR40 siRNA1), 71-4% (GPR40 siRNA2), and 42-1% (GPR40 siRNA3) respectively at 24 h after transfection and were still appreciable to a similar extent at 72 h (Fig. 1C). GPR40 mRNA down-regulation was paralleled by a reduction in GPR40 protein abundance. The percentages of inhibition of GPR40 protein were 69-3% (GPR40 siRNA1), 67-9% (GPR40 siRNA2), and 39-8% (GPR40 siRNA3) respectively at 24 h and remained almost constant at 72 h (Fig. 1B and D). Moreover, transfection reagent alone and control siRNA transfection had no significant effect on the expression of GPR40. Therefore, among the three GPR40 siRNAs, GPR40 siRNA1 was chosen to perform the further experiment.

Effects of GPR40 siRNA on lipoapoptosis of NIT-1 cells

First, the effects of palmitate and oleate on apoptosis of NIT-1 cells were examined. The percentages of apoptotic cells, which were quantified by TUNEL assay and flow cytometric analysis (Annexin V/PI) respectively, were similar. TUNEL assay was performed to detect DNA double-strand breaks in apoptotic cells under a light microscope. Typical TUNEL-positive cells appeared small, intensely stained (brown), and had
fragmented morphology. Flow cytometry analysis (Annexin V/PI) was performed for the purpose of excluding necrotic cells, and detecting the early apoptosis cells. As shown in Fig. 2, NIT-1 cells cultured for 48 h under conditions of 500 μM palmitate showed a remarkable increase in the number of TUNEL-positive cells when compared with the cells cultured in regular medium (control). In parallel, flow cytometry analysis showed that palmitate increased the number of annexin V (C)/PI (K) cells. The percentage of apoptotic cells in the palmitate-treated group was greater than that in control (P<0.01). Oleate showed no significant influence on NIT-1 cells apoptosis (P>O.05 versus control group), whereas after co-incubation with palmitate and oleate (500 μM for each) for 48 h, the percentage of apoptotic cells in GPR40 siRNA-transfected cells was greater than that in mock control (P<0.05). In the cells transfected with control siRNA or mock control, the percentages of apoptotic cells were not significantly different (P>O.05; Fig. 3B–D).

**Role of ERK–MAPK pathway in oleate-promoted inhibition of lipoapoptosis**

To delineate whether phosphatidylinositol 3-kinase (PI3K) and MAPK signaling pathways are involved in oleate-promoted inhibition of β-cell apoptosis induced by palmitate, we examined the activation of Akt, JNK, ERK, and p38 MAPK. As shown in Fig. 4A, the ERK was markedly activated by treatment with oleate and the JNK, p38 MAPK, and Akt were not activated by treatment with oleate, whereas palmitate did not show any positive effects upon Akt, JNK, ERK, and p38 MAPK. Next, we assessed the effects of specific inhibitor for ERK kinase, PD98059. Our data showed that PD98059 nearly completely inhibited the activation of ERK (Fig. 4B). To clarify the role of the ERK–MAPK signaling pathway in the oleate-promoted inhibition of lipoapoptosis, we measured percentages of apoptotic cells indicated among the three groups treated with palmitate (P>0.05; Fig. 3A, C and D). However, after co-incubated with palmitate and oleate (500 μM for each) for 48 h, the percentage of apoptotic cells in GPR40 siRNA-transfected cells was greater than that in mock control (P<0.05). In the cells transfected with control siRNA or mock control, the percentages of apoptotic cells were not significantly different (P>0.05; Fig. 3B–D).
cells in palmitate and oleate co-incubated NIT-1 cells with or without pretreatment with PD98059. As shown in Fig. 5, in the presence of palmitate and oleate, the percentage of apoptotic cells increased when the cells were pretreated with PD98059. Thus, pretreatment with PD098059 significantly suppressed the anti-lipoapoptotic effect of oleate. These results suggested that the activation of ERK pathway, but not JNK, p38MAPK, and Akt pathways, lead to oleate-induced inhibition of lipoapoptosis in NIT-1 cells.

To determine whether Ca\textsuperscript{2+} influx is an important determinant in oleate-stimulated ERK phosphorylation, we performed the L-type Ca\textsuperscript{2+} channel blocker, nimodipine, and PLC inhibitor, U73122. NIT-1 cells were then treated with oleate in the presence or absence of nimodipine or U73122. In contrast to PD98059, preincubation for 30 min with nimodipine or U73122 had no influence on ERK1/2 phosphorylation (Fig. 4B). We next studied the functional role of oleate-induced ERK activation in NIT-1 cells. As shown in Fig. 5B and C, nimodipine or U73122 did not suppress the anti-lipoapoptotic effect of oleate; thus, Ca\textsuperscript{2+} influx is not necessary for oleate-stimulated ERK activation and inhibition of lipoapoptosis.

**Role of ERK–MAPK pathway in GPR40-mediated lipoapoptosis**

To clarify whether ERK–MAPK signaling pathway is involved in GPR40-mediated lipoapoptosis, we reduced the GPR40 expression in NIT-1 cells by siRNA and then measured oleate-induced ERK activation in these cells. Western blotting of phosphoERK1/2 showed that knockdown of GPR40 inhibited the level of ERK1/2 phosphorylation stimulated by oleate (Fig. 6). When compared with the mock cells, the activity of p-ERK was decreased fivefold in GPR40 siRNA-transfected cells. As shown above in Fig. 3B, C, and D, silencing GPR40 significantly inhibited the effects of oleate on lipoapoptosis inhibition. Collectively, it was shown that GPR40 is implicated in the oleate-induced anti-lipoapoptosis probably through the activation of ERK in NIT-1 cells.

We next examined the induction of one of the immediate early genes (IEGs), Egr-1, by FFA stimulation.
in NIT-1 cells. After 30 min of stimulation, Egr-1 was marked activated by oleate, whereas palmitate had little effect (Fig. 7A). This induction was significantly inhibited by pretreatment with ERK kinase inhibitor, PD98059, suggesting that Egr-1 induction by oleate is mainly mediated by ERK activation. In NIT-1 cells transfected with GPR40 siRNA, oleate could not activate Egr-1, suggesting that GPR40 is implicated in the oleate-induced Egr-1 activation (Fig. 7B).

Effect of GPR40 siRNA on FFA-induced increase of insulin secretion

Using NIT-1 cells, we found that at higher glucose concentrations, FFAs induced insulin secretion from NIT-1 cells, suggesting that FFAs amplified glucose-stimulated insulin secretion from NIT-1 cells (Fig. 8A). To test whether inhibition of GPR40 affects FFA-induced insulin secretion, we examined the acute and long-term effects of FFAs (palmitate or oleate) on insulin secretion. FFAs (palmitate or oleate) robustly stimulated insulin secretion from NIT-1 cells transfected with control siRNA or mock but not from cells transfected with GPR40 siRNA (Fig. 8A), although these cells responded to increased glucose levels. The effect of short-term exposure of NIT-1 cells to palmitate or oleate on NIT-1 cells GSIS was similar.

To elucidate whether GPR40 also mediated the long-term negative effect of FFAs on GSIS, we exposed the mock-, control siRNA-, and GPR40 siRNA-transfected cells to palmitate or oleate for 48 h. Mock- and control siRNA-transfected cells in the presence of palmitate or oleate for 48 h resulted in attenuation of GSIS when compared with non-exposed cells. In contrast, insulin secretion from cells transfected with GPR40 siRNA was not impaired by 48 h exposure to palmitate or oleate (Fig. 8B), suggesting that GPR40 is implicated in mediating the long-term negative effect of FFA on GSIS. It is interesting that the insulin secretion of mock- or control siRNA-transfected cells treated with palmitate for a long time was significantly lower than that of cells treated with oleate for a long time; a mixture of both palmitate or oleate partly prevented the palmitate-induced impaired GSIS. After exposure of the GPR40 siRNA-transfected cell to a mixture of both palmitate and oleate for 48 h, the insulin release restored incompletely (Fig. 8B).

Discussion

In this study, we used the mouse insulinoma cell line NIT-1 as models to investigate the β-cell lipoapoptosis. NIT-1, a pancreatic β-cell line established from a
transgenic NOD/Lt mouse, has been shown to display GSIS (Hamaguchi et al. 1991, Soga et al. 2005) and apoptosis induced by various factors (Thomas et al. 2005, Nakamura et al. 2006). These cells are a useful tool in analysis of β-cell function and apoptosis. To determine whether GPR40 is implicated in mediating lipoapoptosis in NIT-1 cells, we used siRNA approach to inhibit the expression of GPR40 in NIT-1 cells. Our results showed that GPR40 expression was inhibited remarkably after treatment with GPR40 siRNA, indicating that the corresponding mRNA sequences for GPR40 siRNA are specific RNAi targets. We employed the saturated fatty acid palmitate (C16:0) and the unsaturated fatty acid oleate (C18:1), since palmitate and oleate are the two most abundant fatty acids in plasma (Brown et al. 2005) and are the potent agonists of GPR40 (Itoh et al. 2003). After 48 h incubation, palmitate induced β-cell apoptosis, whereas oleate prevented NIT-1 cell lipoapoptosis induced by palmitate. These results were consistent with the idea that saturated FFAs are pro-apoptotic and unsaturated FFAs are protective (Eitel et al. 2002, El-Assaad et al. 2003, Maedler et al. 2003). The suggested mechanisms by which FFAs mediate β-cell apoptosis include de novo synthesis of ceramide (Shimabukuro et al. 1998a, b, Lupi et al. 2002a, b, Listenberger et al. 2003, Maedler et al. 2003), NO synthesis (Shimabukuro et al. 1998a, b), suppression of anti-apoptotic factors such as bcl-2 (Shimabukuro et al. 1998a, b, Lupi et al. 2002a, b), accumulation of intracellular triglycerides (Higa et al. 1999, Lupi et al. 2002a, b), generation of ROS (Listenberger et al. 2001), etc. Despite intense investigation, the relative contributions of these various mechanisms to lipoapoptosis in β-cells remain controversial (Busch et al. 2005). Our data demonstrated that knocking down GPR40 expression in NIT-1 cells did not significantly change the percentage of apoptotic cells induced by palmitate. However, the anti-lipoapoptotic activity induced by oleate was suppressed...
by treatment with GPR40 siRNA, suggesting that palmitate-induced β-cell apoptosis might be GPR40 independent, whereas oleate prevents β-cell lipoapoptosis induced by palmitate at least in part through GPR40.

It was shown previously that FFAs promoted the secretion of insulin and the unsaturated FFAs. Oleic acid, linoleic acid, and docosahexaenoic acid stimulated the ERK activation through GPR40, but the activation of ERK is not necessary for insulin secretion (Itoh et al. 2003). As the ERK–MAPK signaling cascade is activated by a wide variety of receptors involved in growth and differentiation, including receptor tyrosine kinase, integrins, and ion channels (Gutkind 1998, Wang et al. 1998), the stimulatory effects of FFAs through GPR40 might evoke other cellular events aside from insulin secretion. PI3K and MAPK are two important signaling molecules, which transduce a variety of external signals, leading to a wide range of cellular responses, including proliferation, differentiation, and apoptosis. In mammals, three major MAPK pathways have been identified: p44/42 MAPK/ERK, SAPK(stress-activated protein kinase)/JNK, and p38 MAPK (Wang et al. 1998). In order to identify the signaling pathways linking the activation of GPR40 to lipoapoptosis, the MAPK and PI3K signaling pathways were studied. We observed that oleate activated ERK; in contrast, palmitate did not show any effects upon ERK phosphorylation. In addition, the protective effect of oleate on lipoapoptosis was suppressed by ERK kinase inhibitor, leading to the idea that the activation of ERK–MAPK pathway is required for oleate-induced anti-lipoapoptotic effects on NIT-1 cells. Moreover, knocking down GPR40 expression resulted in a fivefold decrease in the level of ERK phosphorylation stimulated by oleate. These observations implied that the activation of ERK stimulated by oleate might be GPR40 dependent.

Recently, some studies demonstrated that activation of GPR40 by FFAs causes an increase in intracellular Ca²⁺ levels, which is believed to be via the activation of Gαq-phospholipase C (PLC) pathway with release of Ca²⁺ from the endoplasmic reticulum. The capacity for FFAs to increase cytosolic Ca²⁺ also depends on glucose activation of L-type Ca²⁺ channels and the presence of extracellular Ca²⁺ (Fujinawa et al. 2005, Shapiro et al. 2005). To determine whether these early events are possibly linked to the ERK activation and the anti-lipoapoptosis effect of oleate, we used the L-type Ca²⁺ channel blocker, nimodipine, and PLC inhibitor, U73122, to study the effect on oleate-promoted inhibition of lipoapoptosis and oleate-stimulated ERK activation. We found that an influx of [Ca²⁺]i was not necessary for oleate-induced ERK action and inhibition of lipoapoptosis. Since the induction of immediate early response genes frequently observed concomitant with ERK activation, we examined Egr-1 expression in our model. Egr-1 expression was stimulated by both palmitate and oleate in a time- and dose-dependent manner. Knocking down GPR40 expression also suppressed Egr-1 expression stimulated by both palmitate and oleate. These observations suggest that GPR40 is involved in the regulation of Egr-1 expression in NIT-1 cells.

**Figure 6** Role of ERK–MAPK pathway in GPR40-mediated lipoapoptosis. The mock-, control siRNA-, and GPR40 siRNA-transfected NIT-1 cells were stimulated with oleate (500 μM) for 10 min. Western blotting was performed using anti-phospho- and anti-total kinase antibodies. Data show mean ± S.E.M. of four independent experiments. *P < 0.05 versus mock.

**Figure 7** Egr-1 expression. (A) NIT-1 cells were serum-starved for 2 h with or without pretreatment with PD98059 and treated with 500 μM palmitate or oleate. After 30 min of stimulation, western blotting was performed using anti-Egr antibody. (B) The mock-, control siRNA-, and GPR40 siRNA-transfected cells were serum-starved for 2 h and treated with 500 μM oleate for 30 min. Western blotting was performed. Representative western blotting analysis was shown.
Our results demonstrated that notably c-tional activation of a group of genes termed IEGs, most mitogenic stimuli is the rapid and transient transcriptional activation of genes. A prominent component of the cellular response to physiological levels (Eitel et al. 2002), we examined the induction of one of the immediate Egr-1 by oleate stimulation in NIT-1 cells. Our results demonstrated that Egr-1 induction by oleate is mainly by ERK activation, which is GPR40 dependent. A prominent component of the cellular response to mitogenic stimuli is the rapid and transient transcriptional activation of a group of genes termed IEGs, most notably c-fos and Egr-1 (Ghosh & Greenberg 1995); the patterns of ERK activation and early gene induction by oleate were in accordance with those of oleate-promoted inhibition of lipoapoptosis. Unsaturated FFAs appear to act as an extracellular signaling molecule because they are also protective at very low physiological levels (Eitel et al. 2002), and their effects are potentially mediated by the activation of multiple intracellular signaling pathway. Taken with these results, we proposed the possible mechanism as follows: saturated palmitate induces β-cell apoptosis, which is GPR40 independent. Conversely, oleate, a mono-unsaturated fatty acid, stimulates GPR40 and activates the pathway mediated by ERK–MAPK and increases the expression of Egr-1, leading to the anti-lipoapoptotic effect; [Ca\(^{2+}\)]_i influx is not necessary for this process.

A recent study showed that it failed to detect an increase in apoptosis in GPR40 overexpression mice, which developed diabetes soon after birth (Steneberg et al. 2005). In vivo, GPR40 was activated by a wide range of FFAs including saturated FFAs and unsaturated FFAs which may influence each other with regard to mediating apoptosis. The pancreas mass is continually remodeled in a dynamic process. Apoptosis plays a critical role in modulating expansion and involution of β-cell mass in T2D. Numerous genetic, metabolic, and environmental factors impact this dynamic process (Del Prato et al. 2004). It was found that the rate of cell apoptosis was tenfold greater in lean and threefold greater in obese patients with diabetes when compared with their respective non-diabetic groups (Guilot et al. 2001), suggesting that pancreatic plasticity is an important mechanism to meet the homeostatic demand, and β-cell might protect themselves from lipoapoptosis by adaptive mechanism (Busch et al. 2005). Based on the results described in this study, we concluded that GPR40 might act as the interface between FFAs and β-cell mass plasticity.

FFAs have been reported to modulate insulin secretion. Under short-term exposure, FFAs enhance GSIS, but under long-term exposure they act to attenuate it; an effect called lipotoxicity. Accumulating evidence suggests that GPR40 mediates the majority of the effects of FFA on insulin secretion (Itoh et al. 2003, Salehi et al. 2005, Shapiro et al. 2005, Steneberg et al. 2005, Tomita et al. 2006, Schnell et al. 2007). Our results provide evidence that GPR40 mediated both acute and long-term effects of FFAs on GSIS in NIT-1 cells. On the other hand, the distinct effects of palmitate and oleate on NIT-1 cells insulin secretion are striking. The insulin secretion of control NIT-1 cells treated with palmitate for 48 h was significantly lower than that of cells treated with oleate for 48 h; a mixture of both palmitate and oleate partly prevented the palmitate-induced impaired GSIS. These results were consistent with other previous reports. The long-term negative effect of palmitate on GSIS was possibly accompanied by β-cell apoptosis; conversely, unsaturated fatty acid exhibited the opposite effects, it counteracted the toxic effects of palmitate, and prevented the palmitate-induced impaired GSIS (Maedler et al. 2001, Lupi et al. 2002a,b). The insulin release was not restored completely in GPR40 siRNA cells treated with a mixture...
of both palmitate and olate; possibly, the process is in conjunction with increased β-cell apoptosis.

It has been shown that saturated FFAs reduced the proliferative capacity of β-cells; conversely, unsaturated FFAs exhibited the opposite effects (Maedler et al. 2001). Further studies will be needed to examine the relationship between GPR40 and β-cell proliferation and to determine whether other mechanisms are involved in GPR40-mediated lipoapoptosis under various physiological conditions.

In conclusion, our study provided novel evidence for the action of saturated FFA and unsaturated FFA in β-cells in relation to cell lipoapoptosis and its function. GPR40 is not only a receptor that may participate in the control of insulin secretion by FFA, but it might also play an important role in the control of β-cell apoptosis by unsaturated FFAs. Our data demonstrated that palmitate-induced β-cell lipoapoptosis might not be mediated through GPR40, whereas olate protected NIT-1 cells from palmitate-induced lipoapoptosis, which was mediated at least in part through GPR40. The possible mechanism is as follows: olate promotes the activation of the ERK–MAPK pathway via GPR40 and increases the expression of Egr-1, leading to the anti-lipoapoptotic effect on NIT-1 cells. The results suggested that GPR40 might be implicated in the control of β-cell mass plasticity and GPR40 probably provide a link between obesity and T2D.

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