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Inhibition of GPR40 Protects MIN6 β Cells From Palmitate-Induced ER Stress and Apoptosis

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ABSTRACT

Chronic exposure to elevated concentration of free fatty acids (FFA) has been verified to induce endoplasmic reticulum (ER) stress, which leads to pancreatic β-cell apoptosis. As one of the medium and long chain FFA receptors, GPR40 is highly expressed in pancreatic β cells, mediates both acute and chronic effects of FFA on β-cell function, but the role of GPR40 in FFA-induced β-cell apoptosis remains unclear. In this study, we investigated the possible effects of GPR40 in palmitate-induced MIN6 β-cell apoptosis, and found that DC260126, a novel small molecular antagonist of GPR40, could protect MIN6 β cells from palmitate-induced ER stress and apoptosis. Similar results were observed in GPR40-deficient MIN6 cells, indicating that palmitate-induced β-cell apoptosis is at least partially dependent on ER stress pathway via GRP40. J. Cell. Biochem. 113: 1152–1158, 2012.

KEY WORDS: GPR40 ANTAGONIST; MIN6 β CELLS; PALMITATE; ENDOPLASMIC RETICULUM STRESS; APOPTOSIS

Type 2 diabetes (T2D) is characterized by an increased insulin resistance in peripheral tissues and progressive β-cell failure [Kahn, 2003; Butler et al., 2003a; Donath and Halban, 2004]. Both the defects in insulin secretion and loss of β-cell mass contribute a lot to β-cell failure [Butler et al., 2003a, 2003b; Lupi and Del, 2008; Cnop et al., 2010]. And in obesity-associated diabetes models, the free fatty acids (FFA) have been shown to be cytotoxic to pancreatic β cells, known as lipotoxicity, is one of the main causes of β-cell apoptosis [Butler et al., 2003b; Donath and Halban, 2004; Cnop et al., 2005; Kahn et al., 2006; Lupi and Del, 2008; Thomas et al., 2009].

Acute stimulation of FFA can amplify glucose-stimulated insulin secretion (GSIS) in pancreatic β cells [Briscoe et al., 2003; Itoh and Hinuma, 2005]. However, chronic exposure to elevated levels of FFA, particularly saturated FFA, can cause β-cell dysfunction and induce β-cell apoptosis in T2D [Cnop et al., 2005, 2010]. Recent studies have shown that several events, such as nitric oxide (NO) synthesis and depletion of endoplasmic reticulum (ER) calcium (Ca^{2+}) are associated with lipotoxicity in β cell [Cnop et al., 2001; Robertson et al., 2004; Lupi and Del, 2008]. Nevertheless, the molecular mechanism of FFA-induced β-cell apoptosis is not completely understood.

ER stress is caused by increased misfolded proteins, overloaded chaperones, failure of folding/exporting newly synthesized protein and ER Ca^{2+} depletion. And such conditions induce an impaired folding capacity in ER [Diakogiannaki and Morgan, 2008; van der Kallen et al., 2009; Wang et al., 2009]. Upon initiation of ER stress, the accumulation of unfolded protein triggers unfolded protein response (UPR), which attempts to inhibit protein synthesis, increase folding capacity, promote the degradation of misfolded protein in ER [Harding et al., 2002; Diakogiannaki and Morgan, 2008]. However, when the counterregulatory mechanisms cannot compensate for prolonged ER stress, cell apoptosis finally occurs [Laybutt et al., 2007; Thomas et al., 2010]. More evidences revealed that sustained high level of FFA could cause ER Ca^{2+} depletion, which could induce ER stress of β cells [Cunha et al., 2008; Gwiazda et al., 2009; Cnop et al., 2010]. Pancreatic β cells possess a highly developed ER, required for Ca^{2+} stores, insulin biosynthesis and the folding of newly synthesized proinsulin [Laybutt et al., 2007; Schroder, 2008; Gwiazda et al., 2009; Thomas et al., 2010]. Therefore, β cells are particularly sensitive to ER stress, which is closely related to β-cell apoptosis [Harding and Ron, 2002; Araki et al., 2003; Wang et al., 2009]. Recent studies have indicated that ER stress is strongly associated with palmitate-induced MIN6 β-cell

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apoptosis [Laybutt et al., 2007; Martinez et al., 2008]. Consequently, the mediators of FFA signaling could be important in the ER stress-induced apoptosis of β cells.

G-protein-coupled receptor 40 (GPR40) is highly expressed in pancreatic β cells in both human and rodents, which is identified as one of medium- and long-chain FFA receptors [Briscoe et al., 2003; Itoh and Hinuma, 2005; Hu et al., 2009]. FFA can activate GPR40 to increase cytosolic-free calcium ($[Ca^{2+}]_i$) and amplify GSIS in pancreatic β cells [Schnell et al., 2007]. But sustained high levels of FFA cause β-cell damage, which might lead to abnormalities of cytosolic calcium homeostasis through uninterrupted activation of GPR40 [Abaraviciene et al., 2008b; Gwiazda et al., 2009]. It was reported that GPR40 knockout (KO) mice were resistant to hepatic steatosis, hypertriglyceridemia and some other diabetes-related effects under high fat diet treatments, and GPR40 overexpression in β cells could cause mice to generate a diabetic phenotype [Steneberg et al., 2005]. Above results suggest that overstimulation of GPR40 may be detrimental to β cells in the condition of hyperlipidemia. Accordingly, as one of mediators of FFA signaling, GPR40 may play a crucial role in FFA-induced ER stress and apoptosis in β cells.

This study was specifically designed to investigate a potential role of GPR40 in palmitate-induced MIN6 β-cell apoptosis by both a small molecular antagonist, namely DC260126 [Hu et al., 2009], and a GPR40-knockdown MIN6 cell model. We confirmed that GPR40 is involved in the process of palmitate-induced ER stress and apoptosis in MIN6 cells, and DC260126 could protect palmitate-induced MIN6 cell apoptosis.

MATERIALS AND METHODS

REAGENTS AND MATERIALS

MIN6 cells were kindly provided by Dr. Susumu Seino. All general reagents for cell culture were purchased from GIBCO; palmitate, bovine serum albumin (BSA), and DAPI were purchased from Sigma; and cytokines were obtained from R&D Systems; shRNA matching the mouse GPR40 sequence and negative control shRNA were provided from GenePharma (Shanghai, CN); primers for GPR40 and GAPDH were synthesized by Biosune Biothenology Inc (Shanghai, CN); SYBR Premix ExTaq kits were purchased from Takara; The transfection reagent fugene 6 and cell death ELISA kit were purchased from Roche Diagnostics. Rabbit anti-GAPDH, anti-phospho-eIF2α, anti-eIF2α, anti-cleaved caspase-3 (Asp 175) antibodies, and mouse anti-insulin antibody were purchased from Cell signaling Technology; Rabbit anti-GADD153/CHOP(F-168) antibody and anti-GPR40 (FL-300) antibody were obtained from Santa Cruz Biotechnology. Peroxidase-conjugated Goat anti-rabbit IgG was from Jackson Immuno Research, Alexafluor 488-conjugated goat anti-rabbit IgG and Alexfluor 546-conjugated goat anti-mouse IgG were purchased from Invitrogen.

CELL CULTURE

MIN6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM glucose, 10 mM HEPES, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol at 37°C under 5% CO_2 [Minami et al., 2000].

CELL TRANSFECTION

The short-hairpin RNA (shRNA) of GPR40 was designed to inhibit expression of GPR40 in MIN6 cells. The sequence of GPR40 shRNA were 5′-CACCG CCGTC TCAGT TTCTC CATTT CAAAG AGAAT GGAGA AACTG AGACG GGCTT TTTTG-3′ and 5′-GATCC AAAAA AGCCC GTCTC AGTTT CTCCA TTCTC TTGAA ATGGA GAAAC TGAGA CGGGC-3′. And the vector, PGPU6/GFP/Neo-shNC was used as the negative control (NC) shRNA.

MIN6 cells were seeded in 12-well plates (40,0000 cells per well) for 24 h and transfected with GPR40 shRNA (or negative control shRNA) using fugene 6 transfection reagent with a transfection efficiency more than 80%. Twenty-four hours after transfection, the medium was replaced with growth medium for further experiments.

QUANTITATIVE REAL-TIME PCR (RT-PCR)

RT-PCR was carried out with previous described to examine the transfection efficiency [Hu et al., 2009]. The primers were as follows: mouse GPR40 (forward primer, 5′-GCTTTCCATTGAACTTT-TAGCC-3′, and reverse primer, 5′-CGCTGAGAGCAGCTAGGAAG-3′), GAPDH (forward primer, 5′-CCCACTCCTCCACCTTTGAC-3′, and reverse primer, 5′-CATACCAGGAAATGAGCTTGACAA-3′). The mRNA level was assessed by normalized relative to the amount of GAPDH mRNA.

PALMITATE PREPARATION, CELL TREATMENT, AND APOPTOSIS ASSAY

Palmitate/BSA preparation was carried out as previously described [Martinez et al., 2008]. MIN6 cells were seeded in 12-well plates (40,0000 cells per well) for 24 h. After pretreating the cells with DC260126 for 1 h, 0.4 mM palmitate coupled with 0.5% BSA (palmitate: BSA = 6:1) or cytokines (200 ng/ml IL-1β and 1,000 units/ml IFN-γ) were added into wells. Following a 48 h culture, the apoptotic cells was measured by cell death ELISA kits following its protocol. Briefly, the cells were incubated with lysis buffer. After centrifugation, transfer aliquot of lysate to streptavidin-coated microplate, and incubate lysate with immunoreagent (anti-histone and anti-DNA). Finally add substrate to wells, incubate and measure absorbance at 405 nm.

WESTERN BLOT ANALYSIS

Proteins from each group were collected and the expression levels of proteins were detected using Western blotting as described previously [Hu et al., 2009]. The following primary antibodies and dilutions were used: phospho-eIF2α (1:1,000), eIF2α (1:1,000), GADD153/CHOP (F-168) (1:200), cleaved caspase-3 (Asp 175) (1:500), GPR40 (FL-300) (1:200), GAPDH (1:5,000).

CELL IMMUNOFLUORESCENCE

MIN6 cells were grown on glass coverslips in a six-well plate and incubated with BSA alone, palmitate/BSA or palmitate/BSA mixed with DC260126 (3 μM) for 48 h. Then the cells were fixed in 4% paraformaldehyde for 2 h at room temperature. After fixation the MIN6 cells were incubated with primary antibodies (rabbit anti insulin antibody, dilution 1:100; mouse anti GADD153/CHOP, dilution 1:100) and secondary antibodies (Alexafluor 488-conjugated goat anti-rabbit IgG and Alexfluor 546-conjugated goat

anti-mouse IgG, dilution 1:400). After DAPI staining, coverslips were mounted and captured by fluorescence microscopy (DP70, Olympus).

DATA ANALYSIS

All data are expressed as the mean ± SE. The comparison of different groups was assessed by two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

DC260126 PROTECTS MIN6 CELLS FROM PALMITATE-INDUCED APOPTOSIS

Chronic exposure to elevated saturated FFA levels has been shown to induce pancreatic β-cell apoptosis [Karaskov et al., 2006; Thorn and Bergsten, 2010; Yang et al., 2010]. In order to investigate the role of GPR40 in fatty acid-induced β-cell apoptosis, MIN6 cells were induced apoptosis by palmitate as previously described [Thorn and Bergsten, 2010] and treated with DC260126 at the same time. Sustained palmitate stimulation for 48 h in MIN6 cells induced an obviously raise of apoptosis, and DC260126 could dose-dependently attenuate the effect. Three micromolar DC260126 significantly decreased the palmitate-induced MIN6 cells apoptosis at 48 h (Fig. 1). The results suggested that inhibition of GPR40 can protects MIN6 cells from palmitate-induced apoptosis.

DC260126 RESISTS PALMITATE-INDUCED ER STRESS IN MIN6 CELLS

To identify whether GPR40 is involved in fatty acid-induced ER stress of MIN6 cells, MIN6 cells were incubated with palmitate for 6, 24, and 48 h in the absence or presence of DC260126. The total protein was then collected and the expression levels of some proteins in ER stress pathway (p-eIF2α, total eIF2α, CHOP) and cleaved caspase-3 were analyzed by Western blotting. Figure 2 shows that palmitate causes an increased expression level of p-eIF2α, CHOP and cleaved caspase-3 proteins at 24 and 48 h. However, the increase of ER stress pathways markers p-eIF2α and CHOP were suppressed dose-dependently by DC260126, and 3 μM DC260126 also prevented palmitate-induced enhancive expression of cleaved caspase-3 significantly at both 24 and 48 h (Fig. 2), suggesting that inhibition of GPR40 can protects MIN6 cells from palmitate-induced ER stress.

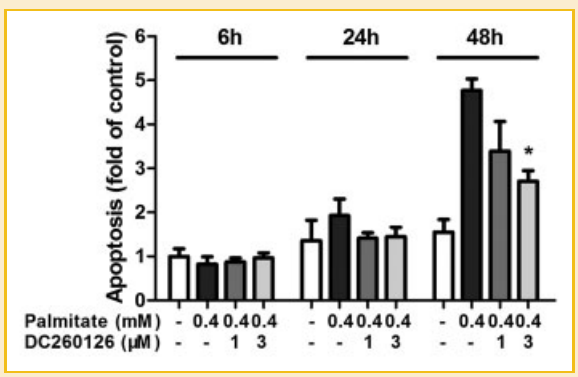

Fig. 1. DC260126 protects palmitate-induced MIN6 cells apoptosis. Apoptosis rate of MIN6 cells was measured by a cell death detection ELISA kit. The relative apoptosis rates were determined at 6, 24, and 48 h after cells were cultured in the absence (white bars) and presence (black bars) of 0.4 mM palmitate, and DC260126 protected MIN6 cells from 0.4 mM palmitate induced apoptosis in a concentration of 1 μM (dark gray bars) or 3 μM (gray bars). $^*P < 0.05$ compared to 0.4 mM palmitate treated alone. Data are shown as mean ± SE of three independent trails each performed with triplicate samples.

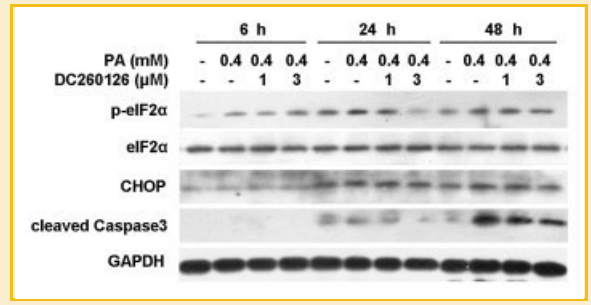

Fig. 2. DC260126 resists palmitate-induced ER stress and apoptosis in MIN6 cells. MIN6 cells were plated in 12-well plates and cultured in the presence of 0.4 mM palmitate with different concentrations of DC260126 (0, 1, 3 μM) for 6, 24, or 48 h, vehicle was 0.5% BSA. Then the cell lysates were collected and subjected to Western blotting using phospho (p)-eIF2α, total eIF2α, CHOP, cleaved caspase-3, and GAPDH antibodies. GAPDH was used as housekeeping protein. Each blot was replicated three times.

MORPHOLOGICAL EVIDENCE OF DC260126 PROTECTS MIN6 CELLS FROM PALMITATE-INDUCED ER STRESS AND APOPTOSIS

In situ distribution of insulin and CHOP proteins were examined by double-immunofluoresence to confirm the key protein in the process of ER stress. There was almost no CHOP-positive cell in negative control MIN6 cells. However, a significant increase of CHOP-stained cells and a slightly decline of insulin expression were observed in cytoplasmic staining in palmitate-treated MIN6 cells. Similar to Western blotting analysis, 3 μM DC260126 could reduce CHOP expression in MIN6 cells under 0.4 mM palmitate-stimulated condition for 48 h (Fig. 3). These data indicate that inhibition of GPR40 can protects MIN6 cells from palmitate-induced ER stress and apoptosis.

DOWNREGULATION OF GPR40 IN MIN6 CELLS BY RNA INTERFERENCE PROTECTS AGAINST PALMITATE-INDUCED ER STRESS AND APOPTOSIS

As shown above, inhibition of GPR40 by DC260126 could counteract palmitate-induced MIN6 cells ER stress and apoptosis. To confirm the protect effect of GPR40 inhibition, the GPR40 shRNA-transfected MIN6 cells were incubated in 48 h palmitate stimulation. GPR40 mRNA and protein expression levels were inhibited (by approximately 50%) in transfected with GPR40 shRNA MIN6 cells compared with negative control (NC) at 24 h (Fig. 4). Then the transfected MIN6 cells were exposure to 0.4 mM palmitate for 48 h. MIN6 cells transfected with GPR40 shRNA were resistant to palmitate-induced apoptosis at 48 h (Fig. 5), and compared with NC group, reduced protein levels of p-eIF2α, CHOP and cleaved caspase-3 were shown in Figure 6. Taken together, these results

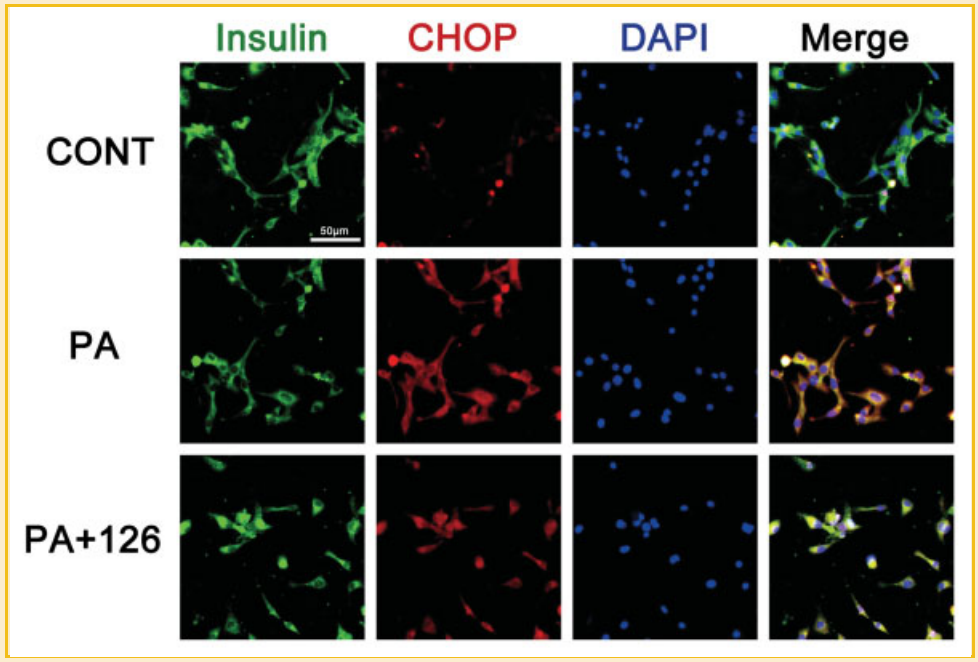

Fig. 3. Change in levels of ER stress marker protein expression and cellular distribution of CHOP in MIN6 cells. MIN6 cells were grew on coverglasses in 12-well plates and cultured in the presence of 0.4 mM palmitate with 3 μM DC260126 for 48 h, vehicle was 0.5% BSA. Cytoplasm staining of insulin and CHOP were marked with green and red fluorescence, respectively, in a double-immunofluorescence experiment. The nuclear was stained with DAPI (blue) and the merge figures were shown on the right. Vehicle, 0.4 mM palmitate and 0.4 mM palmitate co-incubated with 3 μM DC260126-treated cells were shown as negative control (Cont), palmitate (PA) and palmitate + DC260126 (PA + 126). Data were repeated for at least three times, scale bar = 50 μm, and referred to all panels. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

demonstrate that GPR40 has an important role in palmitate-induced MIN6 cells ER stress and apoptosis, and inhibition of GPR40 could be beneficial for resisting palmitate-induced MIN6 cell ER stress and apoptosis.

DC260126 AND DOWNREGULATION OF GPR40 EXPRESSION BY RNA INTERFERENCE COULD NOT PROTECT AGAINST CYTOKINE-INDUCED APOPTOSIS IN MIN6 CELLS

Inflammatory cytokines such as interleukin (IL)-1β, γ-interferon (IFN-γ), and tumor necrosis factor-α (TNF-α) can also induce β-cell apoptosis [Lee et al., 2004; Akerfeldt et al., 2008; Wang et al., 2009]. To determine whether GPR40 mediates cytokine-induced apoptosis in MIN6 cells, MIN6 cells were treated with DC260126 in combination with cytokines (IL-1β 200 ng/ml and IFN-γ 1,000 U/ml) for 6, 24, and 48 h. MIN6 cells transfected with GPR40 shRNA were also treated with cytokines as above. Compared with negative control (no cytokines-treated group), cytokines could induce an apparently apoptosis at 48 h in MIN6 cells. However, neither DC260126 nor GPR40 shRNA-transfected MIN6 cells showed a protective effect in cytokine-treated MIN6 cells (Figs. 7 and 8), indicating that as a FFA receptor, GPR40 selectively mediates FFA-induced MIN6 cells ER stress-related apoptosis.

DISCUSSION

Plasma FFAs levels are increased in obesity-associated T2D [Cnop et al., 2005; Kahn et al., 2006]. Chronically elevated levels of FFA, especially saturated FFA might be cytotoxic to β cells both in vitro and in vivo [Shimabukuro et al., 1998; Cunha et al., 2008; Sachdeva et al., 2009; Giacca et al., 2010]. Several studies have experimentally verified that saturated FFA such as palmitate could induce β-cell apoptosis [Karaskov et al., 2006; Thorn and Bergsten, 2010]. GPR40 is highly expressed in β cells which mediates acutely amplify of GSIS and chronically impair in β cells by medium- and long-chain FFA activation [Briscoe et al., 2003; Abaraviciene et al., 2008a, 2008b; Zhang et al., 2010]. However, whether GPR40 is associated with chronic FFA-induced apoptosis of β cells remains unclear. In this study, we investigated the potential role of GPR40 in FFA-induced MIN6 β-cell apoptosis [Abaraviciene et al., 2008a] have mentioned that GPR40 might be related to palmitate-induced MIN6c4-cell apoptosis in discussion, but no effective evidence has been provided. We found DC260126, a novel antagonist of GPR40, could protect MIN6 cells from palmitate-induced apoptosis at 48 h in a dose-dependent manner (Fig. 1). Suppression of GPR40 expression by RNA interference had also resisted palmitate-induced apoptosis of MIN6 cells (Fig. 5). These results provide the strong evidence that GPR40 is involved in palmitate-induced MIN6 β-cell apoptosis.

Acute stimulation of palmitate could activate GPR40 to induce an increase of $[Ca^{2+}]_i$ to amplify the GSIS in β cells [Briscoe et al., 2003]. However, sustained elevation of $[Ca^{2+}]_i$ by chronic palmitate stimulation leads to a sustained ER Ca^{2+} depletion and reduce the sensitivity of cytosolic Ca^{2+} oscillations to glucose or FFA, which might be the main causes of ER stress in pancreatic β cells [Cunha et al., 2008; Gwiazda et al., 2009; Cnop et al., 2010]. Thus, GPR40 might be of critical importance in the sustained elevation of

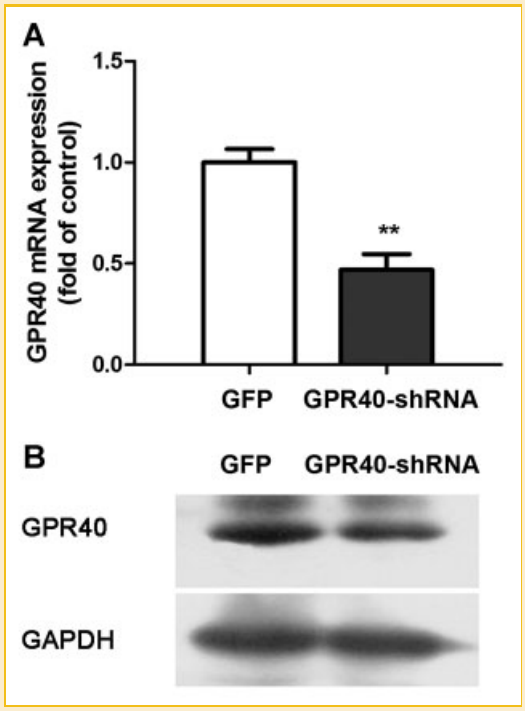

Fig. 4. Expression of GPR40 was inhibited by GPR40 RNA interference in MIN6 cells. A: GPR40-shRNA suppressed the expression of GPR40 mRNA in MIN6 cells to 47% compared to negative plasmid control (GFP) by RT-PCR analysis. $^{**}P < 0.01$, the experiment was carried out for three times. B: Western blot results showed that GPR40-shRNA reduced the expression of GPR40 protein to 50% in MIN6 cells compared to negative plasmid control (GFP), GAPDH was used as housekeeping protein. Each blot was replicated three times.

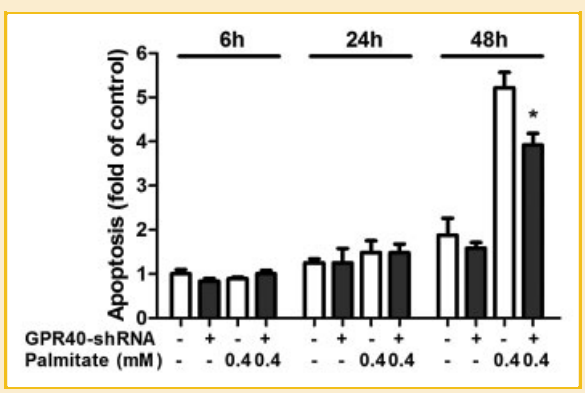

Fig. 5. GPR40 RNA interference reduced palmitate-induced apoptosis in MIN6 cells. MIN6 cells that had been transfected with negative plasmid control (white bars) or GPR40-shRNA (black bars) were stimulated with or without 0.4 mM palmitate. After 6, 24, or 48 h stimulation, the apoptosis rate of MIN6 cells was determined using a cell death detection ELISA kit. Data are shown as percent of the respective control. Values are mean ± SE, the experiments were carried out for three independent times from triplicate samples. $^{*}P < 0.05$ for indicated cells on palmitate treated in GPR40-shRNA transfected cells versus negative control group.

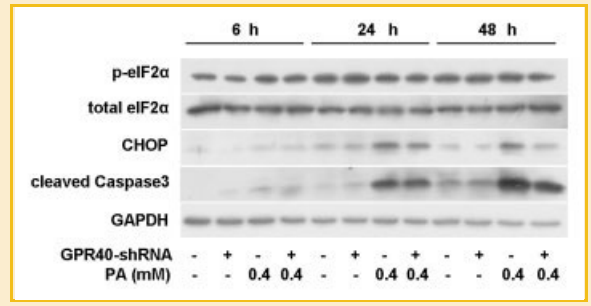

Fig. 6. GPR40 RNA interference resists palmitate-induced ER stress and apoptosis in MIN6 cells. MIN6 cells that had been transfected with negative plasmid control and GPR40-shRNA were cultured with or without 0.4 mM palmitate (PA) for 6, 24 or 48 h, the cell lysates were collected and subjected to Western blotting using phospho(p)-eIF2α, total eIF2α, CHOP, cleaved caspase-3, and GAPDH antibodies. GAPDH was used as housekeeping protein. Blots were replicated three times.

$[Ca^{2+}]_i$-induced ER stress in β cells, which is caused by long-term palmitate stimulation. We had demonstrated that DC260126 could dose-dependently inhibit palmitate-stimulated $[Ca^{2+}]_i$ elevation in MIN6 cells [Hu et al., 2009]. Therefore, we speculated that inhibition of GPR40 in MIN6 cells might provide benefits to suppress ER stress. Hence, the effects of DC260126 treatment or GPR40 knockdown in MIN6 cells on palmitate-induced ER stress were examined.

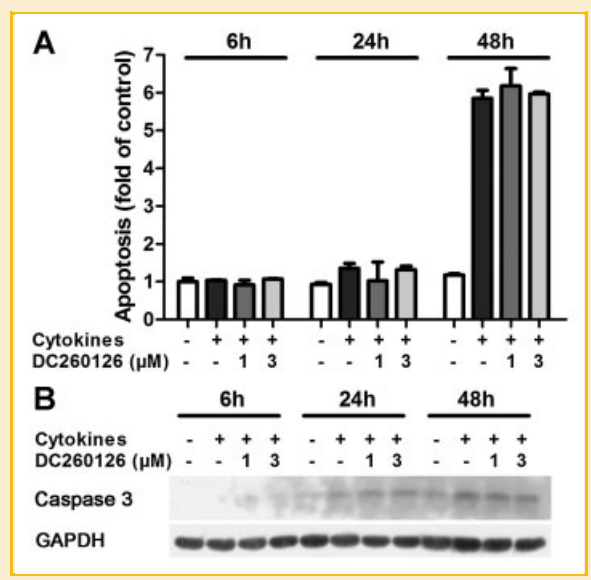

Fig. 7. DC260126 does not protect cytokines induced apoptosis in MIN6 cells. A: The relative apoptosis rates were determined by a cell death detection ELISA kit at 6, 24, or 48 h after cells were cultured in the absence (white bars) and presence (black bars) of cytokines (IL-1β and IFN-γ), and DC260126 in a concentration of 1 μM (dark gray bars) or 3 μM (gray bars) was also co-incubated with cytokines. No significant difference was found between each group. Data were shown as mean ± SE of three independent trails each with triplicate samples. B: Western blot analysis on the expression of cleaved caspase-3 protein from MIN6 cells cultured in the absence of cytokines with 0, 1, or 3 μM DC260126, vehicle was 0.5% BSA. Blots were replicated three times.

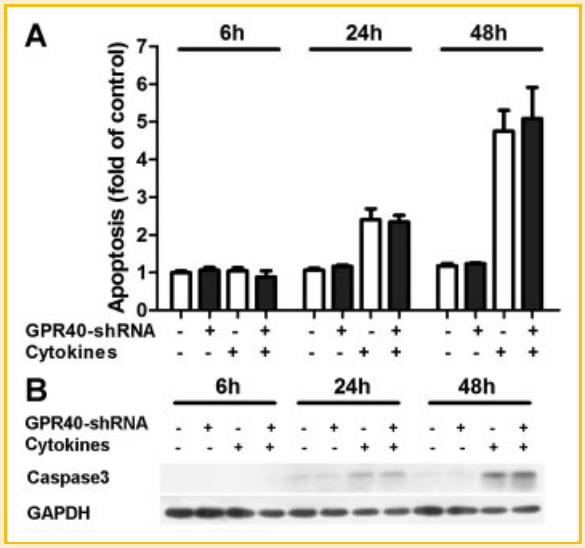

Fig. 8. GPR40 RNA interference does not reduce cytokines induced apoptosis in MIN6 cells. A: MIN6 cells that had been transfected with negative plasmid control (white bars) or GPR40-shRNA (black bars) were stimulated with or without cytokines (IL-1β and IFN-γ). After 6, 24, or 48 h stimulation, the apoptosis rate of MIN6 cells was determined by a cell death detection ELISA kit. Data are shown as percent of the respective control. Values are mean ± SE, the experiments were carried out for three independent times from triplicate samples. B: Western blot analysis on the expression of cleaved caspase-3 protein from cytokines treated GPR40-shRNA or negative plasmid transfected MIN6 cells, vehicle was 0.5% BSA. Blots were replicated three times.

The palmitate-induced ER stress mainly activates PKR-like kinase (PERK) pathway in pancreatic β cells, PERK phosphorylates eukaryotic translation initiation factor2α, leading to inhibition of new protein translation [Diakogiannaki and Morgan, 2008; Cnop et al., 2010; Thomas et al., 2010]. And the proapoptotic transcription factor CHOP, which mediates the lethal effect of PERK signaling, is ubiquitously expressed at a very low level but robustly expressed in β cells under ER stress condition [Oyadomari and Mori, 2004; Wang et al., 2009]. Then cleaved caspase-3 could be activated by PERK pathway and causes β-cell apoptosis [Lei et al., 2008]. Our results confirmed that DC260126 counteracted palmitate-induced upregulation of p-eIF2α, CHOP and cleaved caspase-3 by Western blotting analysis (Fig. 2) and immunofluoresence (Fig. 3). To verify the consequence, we inhibited GPR40 expression in MIN6 cells by RNA interference (Fig. 4), and the experiments of DC260126-treated MIN6 cells were repeated on GPR40-deficient MIN6 cells. As expected, in accord with the effects of DC260126 on MIN6 cells, similar results were observed on GPR40-deficient MIN6 cells (Figs. 5 and 6). The above results provided strong evidences that protective effect of DC260126 in palmitate-induced MIN6 cells apoptosis was at least partially involved in decreased ER stress via GPR40 inhibition. Moreover, it has been elucidated that GPR40 mRNA expression got a down-regulation under long-term FFA stimulation in β cells [Flodgren, 2007]. Although the detailed mechanism still needs further investigation, down-regulation of GPR40 in chronic FFA-stimulated pancreatic β cells might be a compensatory signal that is able to suppress cell death. Consequently, GPR40 play an important positive role in palmitate-induced ER apoptosis in MIN6 β cells, and inhibition of continuous GPR40 activation by GPR40 antagonists or inhibition of GPR40 expression in β cells might provide benefits to prevent FFA-induced β-cell apoptosis.

Compared to FFA-induced apoptosis, inflammatory cytokine-induced apoptosis is another important cause of β-cell apoptosis which is independent of ER stress signaling (Akerfeldt et al., 2008). Cytokines, such as IL-1β, IFN-γ, and TNF-α, are reported to be mediators of β-cell dysfunction and apoptosis [Lee et al., 2004; Akerfeldt et al., 2008]. In this study, we also examined whether DC260126 and GPR40 knockdown were effective to cytokine-induced apoptosis in MIN6 cells. Figures 7 and 8 showed that neither DC260126 nor knocking down GPR40 could protect MIN6 cells from cytokine-induced apoptosis, indicated that GPR40 selectively mediated FFA- but not cytokine-induced MIN6 cell apoptosis.

In summary, the present studies provided supporting evidence that GPR40 mediates palmitate-induced β-cell apoptosis and was at least partially through ER stress pathway. Both a small-molecular antagonist of GPR40, DC260126, and GPR40 knocking-down could protect MIN6 from palmitate-induced apoptosis. Although some GPR40 agonists have been reported to be benefit for T2D treatment as glucose-sensitive insulin secretagogues [Briscoe et al., 2006], and a selective GPR40 agonist, TAK-875 could stimulates glucose-dependent insulin secretion without β-cell toxicity [Tsujihata et al., 2011]. Anyhow, according to our results, using of GPR40 agonist in long-term obesity-associated T2D should be further investigated.

ACKNOWLEDGMENTS

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