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Journal of Cellular Biochemistry 111:634–642 (2010)

Journal of Cellular Biochemistry

SREBP-1c, Pdx-1, and GLP-1R Involved in Palmitate–EPA Regulated Glucose-Stimulated Insulin Secretion in INS-1 Cells

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ABSTRACT

Impairment of glucose-stimulated insulin secretion (GSIS) caused by glucolipotoxicity is an essential feature in type 2 diabetes mellitus (T2DM). Palmitate and eicosapentaenoate (EPA), because of their lipotoxicity and protection effect, were found to impair or restore the GSIS in beta cells. Furthermore, palmitate was found to up-regulate the expression level of sterol regulatory element-binding protein (SREBP)-1c and down-regulate the levels of pancreatic and duodenal homeobox (Pdx)-1 and glucagon-like peptide (GLP)-1 receptor (GLP-1R) in INS-1 cells. To investigate the underlying mechanism, the lentiviral system was used to knock-down or over-express SREBP-1c and Pdx-1, respectively. It was found that palmitate failed to suppress the expression of Pdx-1 and GLP-1R in SREBP-1c-deficient INS-1 cells. Moreover, down-regulation of Pdx-1 could cause the low expression of GLP-1R with/without palmitate treatment. Additionally, either SREBP-1c down-regulation or Pdx-1 over-expression could partially alleviate palmitate-induced GSIS impairment. These results suggested that sequent SREBP-1c-Pdx-1-GLP-1R signal pathway was involved in the palmitate-caused GSIS impairment in beta cells. J. Cell. Biochem. 111: 634–642, 2010.

KEY WORDS: TYPE 2 DIABETES MELLITUS; GLUCOSE-STIMULATED INSULIN SECRETION; STEROL REGULATORY ELEMENT-BINDING PROTEIN-1C; PANCREATIC AND DUODENAL HOMEOBOX-1; GLUCAGON-LIKE PEPTIDE-1 RECEPTOR

Type 2 diabetes mellitus (T2DM) is a common metabolic disorder and the pathogenesis involves two core defects, that is, the insulin resistance and impaired beta cell function [Martin et al., 1992; Weyer et al., 1999]. Impaired glucose-stimulated insulin secretion (GSIS) is an essential feature of beta cell dysfunction, which is widely believed to be secondary to the prolonged exposure to high glucose and lipid levels termed "glucolipotoxicity." The molecular mechanism of glucolipotoxicity has been extensively explored [Relimpio, 2003].

Many effectors have been found to be involved in the GSIS regulation including sterol regulatory element-binding protein (SREBP)-1c, insulin promoter factor (IPF)-1/pancreatic and duodenal homeobox (Pdx)-1, and glucagon-like peptide (GLP)-1 receptor (GLP-1R).

SREBP-1c is a member of the membrane-bound transcription factor basic helix-loop-helix (bHLH) leucine zipper family. Conventionally, SREBP-1 is viewed as a nutritional regulator of lipogenic enzymes in the liver [Shimano et al., 1999; Shimano, 2007]. It is up-regulated by dietary intake of carbohydrates, sugars, and saturated fatty acids and down-regulated by polyunsaturated fatty acids such as eicosapentaenoate (EPA). This type of nutritional SREBP-1c regulation has recently been observed in both cultured beta cells and isolated islets of mice [Kato et al., 2008]. More importantly, activation of SREBP-1c in pancreatic beta cells has been shown to be involved in insulin secretion impairment [Kakuma et al., 2000; Diraison et al., 2004].

Pdx-1 is one of the most reported transcription factors that are critical to both beta cell development and function. It is known that Pdx-1 plays an essential role in the regulation of beta cell neogenesis, differentiation, and apoptosis [Johnson et al., 2003]. In addition, Pdx-1 has been reported to be one of a few genes associated with an autosomal dominant form of diabetes-maturity-onset diabetes of the young (MODY)-4 in humans [Stoffers et al., 1997; Habener and Stoffers, 1998]. Additionally, specific removal of Pdx-1 in mice caused a severe diabetic phenotype due to beta cell dysfunction [Gannon et al., 2008]. Based on these findings, Pdx-1,

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Received 20 April 2010; Accepted 8 June 2010 • DOI 10.1002/jcb.22750 • © 2010 Wiley-Liss, Inc.
Published online 29 June 2010 in Wiley Online Library (wileyonlinelibrary.com).

besides its function in beta cell differentiation, is also important as a regulator of beta cell function [Keller et al., 1986; Johnson et al., 2006].

GLP-1 is a 30-amino acid peptide and formed in the L cells [Nauck et al., 1986]. GLP-1 is a powerful incretin which could enhance the GSIS, regulate the growth of beta cell mass, delay gastric emptying, and inhibit glucagon secretion [Holst, 2007]. It exerts these effects after the peptides reach the targeted cells and thereafter bind to their receptors—the GLP-1R. GLP-1R is a member of the seven transmembrane family of G protein-coupled receptors (7TMRs) and mediates a cascade of events triggered by GLP-1.

Taken together, these three effectors (SREBP-1c, Pdx-1, and GLP-1R) could regulate the GSIS, respectively. However, it is still unclear whether they act in different signal pathways or their actions are connected in an integrated network. Recently, we found that palmitate and EPA could impair or restore the GSIS in INS-1 cells. Additionally, the expression levels of SREBP-1c, Pdx-1, and GLP-1R were affected by palmitate. In the current studies, we regulated the expression level of SREBP-1c and Pdx-1 in INS-1 cells through lentiviral system to analyze the role and relationship of SREBP-1c, Pdx-1, and GLP-1R in palmitate/EPA involved GSIS regulation. This study would help to understand the possible mechanisms involved in the beta cell dysfunction caused by lipotoxicity and would be useful in disease prevention for T2DM.

MATERIALS AND METHODS

TREATMENT OF INS-1 CELLS WITH PALMITATE AND EPA

INS-1 cells (Passages 48–58) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (v/v), 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and 1 mM pyruvate at 37°C in humidified air with 5% CO_2 [Asfari et al., 1992]. Culture medium was changed every 48 h. Palmitate and EPA (Sigma-Aldrich, St. Louis, MO) were dissolved in RPMI 1640 containing 0.5% bovine serum albumin (BSA) (Calbiochem Merck, Germany) to a final concentration of 400 μmol (palmitate) and 50 μmol (EPA), respectively. INS-1 cells were treated for 48 h before the indicated experiments.

CONSTRUCTION OF RECOMBINANT LENTIVIRAL VECTORS CONTAINING miRNA-Pdx-1 AND miR RNAi-SREBP-1c

miRNA oligonucleotides targeting at Pdx-1 and SREBP-1 were designed using the BLOCK-iT™ RNAi designer (Invitrogen Corporation, CA). The sequence of miRNA targeting at Pdx-1 was CATG AATAGTGAGGAGCAGTA. The sequence of miRNA targeting at SREBP-1c was CCCTAACAGACTGACACTGAT. Oligonucleotides encoding the miRNA sequences were cloned into a pcDNA™ 6.2-GW/miR expression vector (Invitrogen Corporation). BP recombination between pcDNA™ 6.2-GW/miR and pDONR™221 was carried out, followed by LR recombination between pDONR™221 and pLenti6/V5-DEST vector, as described in the BlockiT miRNA Lentivirus expression system manual (Invitrogen Corporation). The sequences of plasmid pLenti-miRNA-SREBP-1c-KD and pLenti-miRNA-Pdx-1-KD were verified by DNA sequencing (1st base, Singapore). pLenti6/V5-DEST vector was used as the control plasmid.

CONSTRUCTION OF RECOMBINANT LENTIVIRAL VECTORS CONTAINING Pdx-1 cDNA

Rat Pdx-1 gene was subcloned as a 1.4-kb *Bam*H1 and *Xho*1 fragment into pENTR3C (Invitrogen Corporation). The LR recombination between pENTR3C and pLenti6/V5-DEST vector was carried out, as described in the Lentivirus gateway expression system manual. The sequences of plasmid pLenti-Pdx-1-OE were verified by DNA sequencing (1st base, Singapore). pLenti6/V5-DEST vector was used as control plasmid.

LENTIVIRUS PRODUCTION

For each reaction, lentiviral vectors and lipofectamine 2000 reagent (Invitrogen Corporation) were first diluted separately in opti-MEM I Reduced Serum Medium (Invitrogen Corporation) and then mixed together followed by incubation at room temperature for 20 min. 293FT cells (Invitrogen Corporation) were resuspended and mixed with DNA-Lipofectamine 2000 complexes. After overnight incubation in a 37°C incubator, the medium was removed and the virus was harvested after 48–72 h.

TRANSIENT OVER-EXPRESSION AND KNOCK-DOWN OF Pdx-1 AND SREBP-1c

INS-1 cells were plated at 25% confluence and virus was added gently at fixed titer. After overnight incubation in a 37°C incubator, the medium was replaced with complete cell culture medium. After 48–72 h, cells were processed and the knock-down/over-expression levels were evaluated.

REAL-TIME POLYMERASE CHAINS REACTION (PCR)

Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. cDNA was synthesized through reverse transcription PCR (Promega Corporation, Madison, WI). SYBR green master (Applied Biosystems, Foster city, CA), forward and reverse primers, and cDNA template were mixed together thoroughly. Quantitative results were generated by the 7500 Fast System SDS software. The relative gene expression levels were calculated compared to that of β-actin. Forward and reverse primers for β-actin were 5′-GCTCTTTTCC-AGCCTTCCTT-3′ and 5′-CTTCTGCATCCTGTCAGCAA-3′; forward and reverse primers for SREBP-1c were 5′-GCGGAGCCATGGATTG-CAC-3′ and 5′-CTCTTCCTTGATA CCAGGCCC-3′; forward and reverse primers for Pdx-1 were 5′-GGACATCTCCCCATACGAAG-3′ and 5′-CGTTGTCCCGCTACTACG TT-3′; forward and reverse primers for GLP-1R were 5′-CCGGGTCATCTGCATCGT-3′ and 5′-AGTCTG-CATTTGATGTCGGTCTT-3′.

IMMUNOBLOTTING ANALYSIS

Cells were incubated in 12-well plates for 72 h and then harvested in homogenizing buffer by cell-lifters. Lysates were sonicated on ice. Aliquots of samples (40 μg protein each) were loaded on 10% SDS–PAGE gels for electrophoresis and electrotransferred to polyvinylidene difluoride membranes. Membranes were blocked with rabbit anti-SREBP-1-c (1:5,000) antibody (Bethyl, Montgomery, TX), anti-Pdx-1 (1:1,500) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GLP-1R (1:2,000) antibody (Santa Cruz Biotechnology) at 4°C overnight, respectively. After washes, membranes were

incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,500) (Santa Cruz Biotechnology) for 1 h. The chemiluminescent signals were detected using an ECL system (Pierce Chemical Co., Rockford, IL) following manufacturer's instructions.

MEASUREMENT OF INSULIN SECRETION

INS-1 cells were seeded in 24-well culture plates followed by incubating in 1 ml of KRBH buffer (124 mM NaCl, 5.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM Hepes, pH 7.4) with 0.1% BSA at 37°C for 30 min. Thereafter, cells were incubated with KRBH buffer containing 2.8 and 16.7 mM glucose for another 30 min at 37°C. 0.9 ml of KRBH buffer in each well was collected and then diluted with RIA buffer. 0.9 ml of acid–ethanol was added for insulin content detection. Triplicates were performed for each independent experiment. RIA kits were used to determine the amount of secreted insulin and the insulin content (Linco, Research, St. Charles, MO). Results of insulin secretion were expressed as percentages of insulin content.

STATISTICAL ANALYSIS

The results are expressed as mean ± SEM and analyzed by two-tail *t*-test or ANOVA. $P < 0.05$ was considered as significant.

RESULTS

PALMITATE AND EPA REGULATE GSIS AND THE EXPRESSION LEVELS OF SREBP-1c, Pdx-1, AND GLP-1R IN NORMAL (SREBP-1c-N) INS-1 CELLS

To investigate the effect of palmitate and EPA on beta cell function, insulin secretion was detected in normal INS-1 cells (SREBP-1c-N). As shown in Figure 1A, 16.7 mM glucose failed to induce insulin release in palmitate-treated SREBP-1c-N cells (0.89 ± 0.10 at 16.7 mM Glc vs. 0.84 ± 0.13 at 2.8 mM Glc, $n = 5$, $P > 0.05$). Conversely, when cells were incubated in palmitate supplement with EPA (PA–EPA group), they could normally release insulin and there was no significant difference between control and palmitate–EPA-treated cells (control: 1.85 ± 0.08 vs. PA–EPA: 1.72 ± 0.12, $n = 5$, $P > 0.05$). This indicated that the negative effect of palmitate on insulin secretion was nullified by EPA. Additionally, EPA individually did not affect the insulin secretion (control: 1.85 ± 0.08 vs. EPA: 1.75 ± 0.16, $n = 5$, $P > 0.05$). Together, these results revealed that palmitate could inhibit and EPA could restore the insulin secretion on glucose stimulation.

Since SREBP-1c, Pdx-1, and GLP-1R were found to be important for the GSIS, the expression levels of these three genes was investigated. According to the real-time PCR, SREBP-1c was found to be

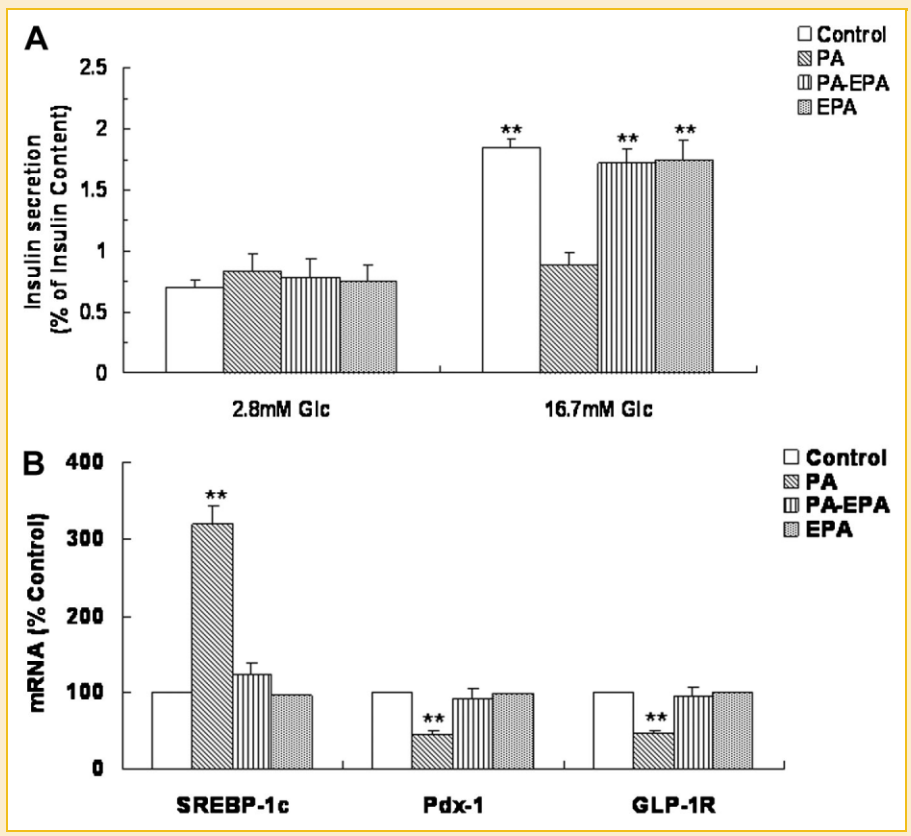

Fig. 1. Insulin secretion and gene expression in normal (SREBP-1c-N) INS-1 cells treated without (control) or with palmitate (PA), palmitate–EPA (PA–EPA), or EPA. A: INS-1 cells were incubated with low glucose (2.8 mmol/L Glc) and high glucose (16.7 mM Glc). Secreted insulin was measured and expressed as % of insulin content. B: Levels of mRNA of SREBP-1c, Pdx-1, and GLP-1R genes were determined by real-time PCR. mRNA quantities were calculated as a ratio to the β-actin level. In each cDNA sample, data are shown as the relative expression ratio to control samples. Five independent experiments were performed using four sets of INS-1 cells, and results are expressed as means ± SEM. $^{**}P < 0.01$ versus control 2.8 mM Glc group in A and $^{**}P < 0.01$ versus control SREBP-1c group in B.

highly up-regulated by palmitate (PA group) (320.0 ± 23.0% vs. control, n = 5, $P < 0.01$) (Fig. 1B). The similar result was found at the protein level through the Western blotting analysis (Fig. 4A). There was no significant change of SREBP-1c expression level in both the PA–EPA and EPA groups (Fig. 1B). On the contrary, the expression levels of Pdx-1 and GLP-1R were significantly down-regulated after treated with palmitate at mRNA levels (44.0 ± 6.0% and 46.3 ± 4.0% vs. control, n = 5, $P < 0.01$) and protein levels (Figs. 1B and 4B,C). This down-regulation was nullified by the supplement of EPA (Fig. 1B). These data indicated that palmitate up-regulated SREBP-1c and down-regulated Pdx-1 and GLP-1R in normal INS-1 cells. EPA could suppress this effect.

PALMITATE AND EPA REGULATE GSIS AND THE EXPRESSION LEVELS OF SREBP-1c, Pdx-1, AND GLP-1R IN SREBP-1c KNOCK-DOWN (SREBP-1-KD) INS-1 CELLS

Lentiviral system was used to knock-down SREBP-1c in INS-1 cells. It was found that the mRNA level of SREBP-1c in SREBP-1c-KD cells (control group) was significantly decreased by around 71.0% (n = 5, $P < 0.01$) (Table I). The similar result was found at the protein level (Fig. 4A). Additionally, the SREBP-1c was markedly down-regulated in PA, PA–EPA, and EPA groups. These data showed that lentiviral system could efficiently knock-down SREBP-1c and this was not affected by PA and EPA treatment.

Insulin secretion in SREBP-1c-KD cells was detected to identify the role of SREBP-1c on palmitate/EPA involved GSIS regulation. As shown in Figure 2, basal secretion was not affected by SREBP-1 deficiency. It has been shown that palmitate impaired GSIS in normal INS-1 cells (Fig. 1A). Dissimilarly, SREBP-1-KD cells could still release insulin on glucose stimulation when treated by palmitate (SREBP-1-KD: 1.49 ± 0.10 vs. SREBP-1-N: 0.89 ± 0.10, n = 5, $P <$ 0.01). There was no significant difference of insulin secretion between these two cells in the EPA and EPA–PA groups. These results indicated that the absence of SREBP-1c suppressed palmitate-induced GSIS impairment.

Furthermore, the expression levels of Pdx-1 and GLP-1R were estimated in SREBP-1c-KD cells as well. SREBP-1c knock-down did not affect the expression of Pdx-1 and GLP-1R in control groups (Table I and Fig. 4B,C). Although palmitate could still induce the down-regulation of Pdx-1 and GLP-1R in SREBP-1c-KD cells, the extent was markedly decreased. No obvious effect of EPA on Pdx-1 and GLP-1R was observed (Table I). This result showed that palmitate had a smaller impact on suppressing the expression levels of Pdx-1 and GLP-1R in SREBP-1c knock-down cells than that in normal INS-1 cells.

THE EXPRESSION LEVELS OF SREBP-1c, Pdx-1, AND GLP-1R IN Pdx-1 KNOCK-DOWN (Pdx-1-KD) AND OVER-EXPRESSED (Pdx-1-OE) INS-1 CELLS

To further investigate the relationship of SREBP-1c, Pdx-1, and GLP-1R in palmitate/EPA cascade, Pdx-1 was knocked down (Pdx-1-KD) and over-expressed (Pdx-1-OE) through lentiviral system. Real-time PCR showed that Pdx-1 could be significantly knocked down by ~70% (Table II). The immunoblotting analysis showed the similar result at the protein level (Fig. 4B). It was found that SREBP-1c expression was similar in Pdx-1-N and -KD cells among the various treatment groups, indicating that Pdx-1 has no effect on the expression of SREBP-1c. Conversely, GLP-1R was down-regulated by around 55% (n = 5, $P < 0.01$) at mRNA level (Table II) and protein level (Fig. 4C) in Pdx-1-KD cells (control group). This result demonstrated that Pdx-1 could regulate the expression of GLP-1R. Moreover, the GLP-1R expression level was decreased in PA, PA–EPA, and EPA groups with similar extent in Pdx-1-KD cells, which indicated that EPA failed to restore the palmitate-induced GLP-1R down-regulation in Pdx-1 deficiency cells.

Furthermore, as shown in Table III, Pdx-1 was over-expressed by approximately twofolds through lentiviral system (control group) at mRNA level. The Western blotting analysis presented the similar result as well (Fig. 4B). Palmitate and/or EPA were added in INS-1 cells after 48 h incubation with lentivirus and the expression levels of SREBP-1c and GLP-1R were determined. In Pdx-1-OE cells, the expression of SREBP-1c displayed similar pattern as that in Pdx-1-N cells, which further demonstrated that Pdx-1 did not regulate the expression of SREBP-1c. Additionally, the expression of GLP-1R was not affected by the transient Pdx-1 over-expression in control groups. However, a marked variation of GLP-1R expression between Pdx-1-N and -OE cells was exhibited in PA groups (Table III and Fig. 4C). These results demonstrated that palmitate failed to

TABLE I. Gene Expression in SREBP-1c Knock-Down (SREBP-1c-KD) INS-1 Cells Treated Without (Control) or With Palmitate (PA), Palmitate–EPA (PA–EPA), or EPA

	Control		PA		PA–EPA		EPA	
	SREBP-1c-N	SREBP-1c-KD	SREBP-1c-N	SREBP-1c-KD	SREBP-1c-N	SREBP-1c-KD	SREBP-1c-N	SREBP-1c-K
SREBP-1c	100	$29.0 \pm 3.0^{**,\#}$	$320.0 \pm 23.0^{**}$	$35.0 \pm 2.5^{**,\&}$	123.0 ± 25.0	$32.0 \pm 3.1^{**,\wedge}$	97.0 ± 5.0	$31.0 \pm 2.8^{**,\$}$
Pdx-1	100	102.0 ± 8.3	$44.0 \pm 6.5^{**}$	$78.9 \pm 5.0^{**,\&}$	92.0 ± 12.2	96.0 ± 10.0	98.0 ± 14.0	100.5 ± 9.4
GLP-1R	100	98.5 ± 11.0	$46.3 \pm 4.2^{**}$	$74.8 \pm 2.1^{**,\&}$	94.0 ± 13.7	96.0 ± 9.4	97.0 ± 15.0	97.5 ± 8.3

mRNA levels of SREBP-1c, Pdx-1, and GLP-1R genes were determined by real-time PCR. mRNA quantities were calculated as a ratio to the β-actin level. In each cDNA sample, data are shown as the relative expression ratio to control samples from SREBP-1c-N INS-1 cells. Five independent experiments were performed using four sets of INS-1 cells, and results are expressed as means ± SEM.
$^{**}P < 0.01$ versus control SREBP-1c-N group. $^{\#}P < 0.01$ versus control SREBP-1c-N group. $^{\&}P < 0.01$ versus PA SREBP-1c-N group. $^{\wedge}P < 0.01$ versus PA–EPA SREBP-1c-N group. $^{\$}P < 0.01$ versus EPA SREBP-1c-N group.

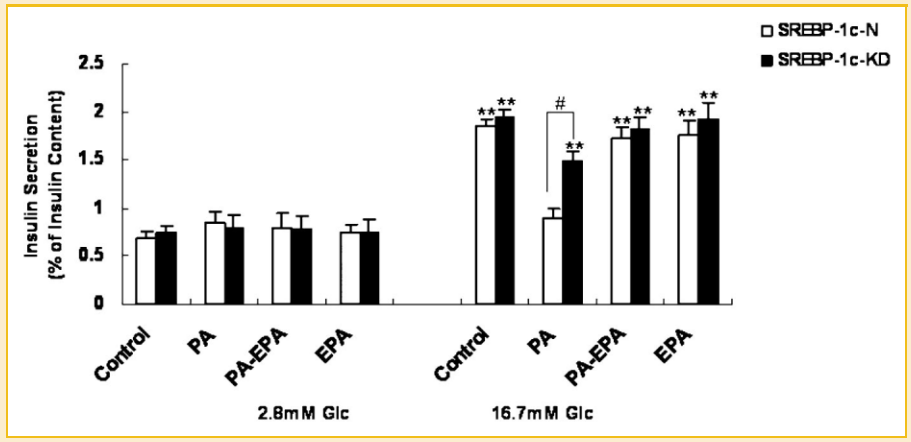

Fig. 2. Insulin secretion in SREBP-1c knock-down (SREBP-1c-KD) INS-1 cells treated without (control) or with palmitate (PA), palmitate–EPA (PA–EPA), or EPA. INS-1 cells were incubated in low Glc (2.8 mmol/L) and high Glc (16.7 mM). Secreted insulin was measured and expressed as % of insulin content. Five independent experiments were performed using four sets of INS-1 cells, and results are expressed as means ± SEM. $^{**}P < 0.01$ versus control SREBP-1c-N group; $^{\#}P < 0.01$.

down-regulate the expression of GLP-1R when Pdx-1 was normally expressed. Nevertheless, up-regulation of Pdx-1 could not augment the GLP-1R expression.

PALMITATE AND EPA REGULATE THE GSIS IN Pdx-1-KD AND Pdx-1-OE INS-1 CELLS

To investigate the effect of Pdx-1 on beta cell function in palmitate–EPA cascade, insulin secretion was detected in Pdx-1-KD and Pdx-1-OE cells. In Figure 3A, Pdx-1 knock-down markedly suppressed the insulin secretion in control group (0.80 ± 0.04 at 2.8 mM Glc vs. 0.90 ± 0.06 at 16.7 mM Glc, n = 5, $P > 0.05$). The same results were found in PA, PA–EPA, and EPA groups as well. These results indicated that Pdx-1 deficiency abolished the restoration effect from EPA. As for Pdx-1-OE cells, insulin secretion could be induced by high glucose in PA group (Pdx-1-OE: 1.79 ± 0.05 at 16.7 mM Glc vs. 0.87 ± 0.04, n = 5, $P < 0.01$), which indicated that the palmitate-induced GSIS impairment was significantly blunted when the expression of Pdx-1 was not decreased. Besides, both Pdx-1-OE and -N cells displayed the similar GSIS patterns among control, PA–EPA, and EPA groups. Taken together, it could be considered that Pdx-1

TABLE II. Gene Expression in Pdx-1 Knock-Down INS-1 Cells (Pdx-1-KD) INS-1 Cells (Pdx-1-OE) Treated Without (Control) or With Palmitate (PA), Palmitate–EPA, or EPA

	Control		PA		PA–EPA		EPA	
	Pdx-1-N	Pdx-1-KD	Pdx-1-N	Pdx-1-KD	Pdx-1-N	Pdx-1-KD	Pdx-1-N	Pdx-1-KD
SREBP-1c	100	102.0 ± 9.0	318.0 ± 21.5**	330.0 ± 18.3**	119.0 ± 12.7	122.0 ± 25.5	95.4 ± 9.0	97.5 ± 6.3
Pdx-1	100	31.4 ± 4.2$^{**,\#}$	44.0 ± 6.5**	38.0 ± 2.50**	93.8 ± 8.3	29.3 ± 4.5$^{**,\&}$	96.5 ± 12.0	30.6 ± 3.4$^{**,\wedge}$
GLP-1R	100	45.2 ± 3.1$^{**,\#}$	52.0 ± 8.2**	40.6 ± 5.1**	95.5 ± 10.2	42.0 ± 4.1$^{**,\&}$	98.0 ± 13.4	43.4 ± 3.6$^{**,\wedge}$

mRNA levels of SREBP-1c, Pdx-1, and GLP-1R genes were determined by real-time PCR. mRNA quantities were calculated as a ratio to the β-actin level in the each cDNA sample. Data are shown as the relative expression ratio to control samples (Pdx-1-N). Five independent experiments were performed using four sets of INS-1 cells, and results are expressed as means ± SEM.
$^{**}P < 0.01$ versus control Pdx-1-N group. $^{\#}P < 0.01$ versus control Pdx-1-N group. $^{\&}P < 0.01$ versus PA–EPA Pdx-1-N group. $^{\wedge}P < 0.01$ versus EPA Pdx-1-N group.

TABLE III. Gene Expression in Pdx-1 Over-Expression INS-1 Cells (Pdx-1-OE) Treated Without (Control) or With Palmitate (PA), Palmitate–EPA, or EPA

	Control		PA		PA–EPA		EPA	
	Pdx-1-N	Pdx-1-OE	Pdx-1-N	Pdx-1-OE	Pdx-1-N	Pdx-1-OE	Pdx-1-N	Pdx-1-OE
SREBP-1c	100	98.5 ± 8.4	310.3.0 ± 14.5**	332.0 ± 16.8**	117.0 ± 19.0	120.4 ± 24.0	98.5 ± 11.0	104.5 ± 6.0
Pdx-1	100	292.0 ± 12.5$^{**,\#}$	42.0 ± 8.4**	183.0 ± 26.2$^{**,\&}$	94.7 ± 9.0	279.6 ± 19.0$^{**,\wedge}$	95.1 ± 10.0	283.5 ± 7.2$^{**,\$}$
GLP-1R	100	121.0 ± 25.5	55.0 ± 6.8**	108.6 ± 9.0$^{\&}$	96.3 ± 11.0	116.0 ± 18.0	96.5 ± 16.9	113.0 ± 15.5

mRNA levels of SREBP-1c, Pdx-1, and GLP-1R genes were determined by real-time PCR. mRNA quantities were calculated as a ratio to the β-actin level in the each cDNA sample. Data are shown as the relative expression ratio to control samples (Pdx-1-N). Five independent experiments were performed using four sets of INS-1 cells, and results are expressed as means ± SEM.
$^{**}P < 0.01$ versus control Pdx-1-N group. $^{\#}P < 0.01$ versus control Pdx-1-N group. $^{\&}P < 0.01$ versus PA Pdx-1-N group. $^{\wedge}P < 0.01$ versus PA–EPA Pdx-1-N group. $^{\$}P < 0.01$ versus EPA Pdx-1-N group.

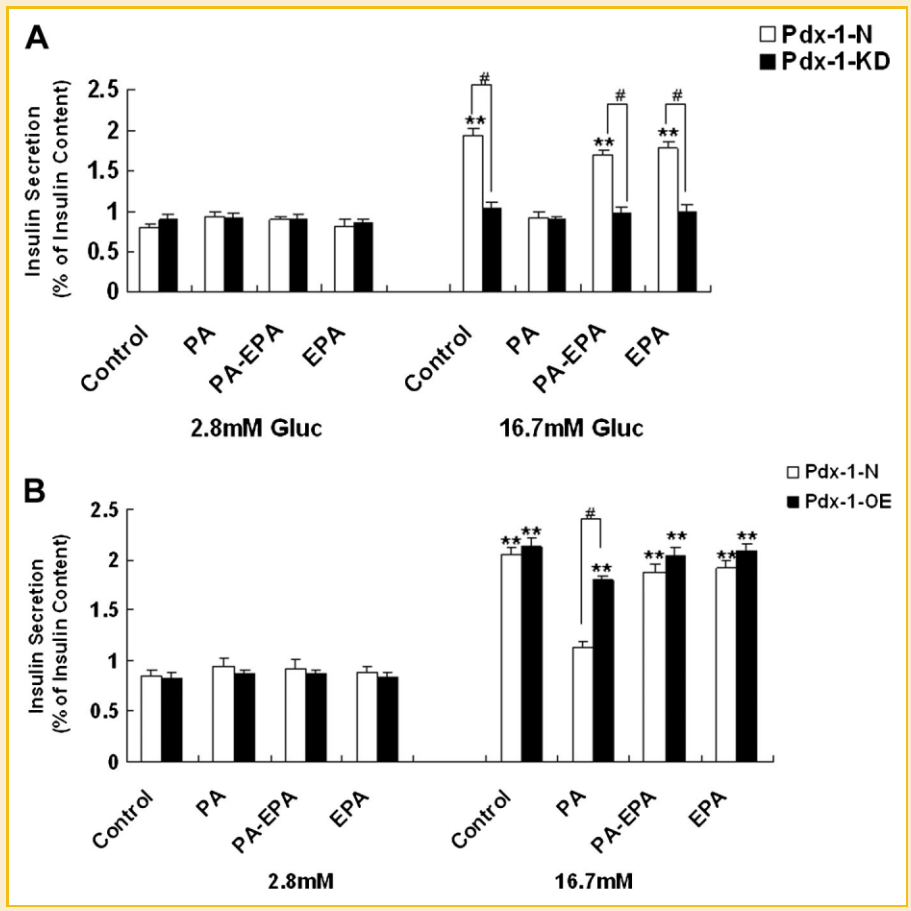

Fig. 3. Insulin secretion in Pdx-1-KD (A) and Pdx-1-OE INS-1 cells (B) treated without (control) or with palmitate (PA), palmitate-EPA (PA-EPA), or EPA. INS-1 cells were incubated in low Glc (2.8 mmol/L) and high Glc (16.7 mM). Secreted insulin was measured and expressed as % of insulin content. Five independent experiments were performed using four sets of INS-1 cells, and results are expressed as means ± SEM. $^{**}P < 0.01$ versus control Pdx-1-N group; $^{\#}P < 0.01$.

over-expression could nullify the palmitate-induced GSIS impairment although it failed to enhance the insulin secretion.

DISCUSSION

It is well known that beta cell dysfunction is involved in the development of T2DM. The long-term exposure to a high lipid condition plays a vital role in the impairment of beta cell function. Palmitate, as a commonly used saturated fatty acid, could blunt GSIS due to its lipotoxicity [Kahn, 2001; El Assaad et al., 2003]. Our current studies demonstrated that palmitate could cancel the glucose-induced insulin release in INS-1 cells (Fig. 1A). This palmitate-caused GSIS impairment was restored by a supplement of EPA. However, EPA itself had no effect on GSIS regulation, indicating that EPA did not intrinsically improve the GSIS but canceled the palmitate-caused GSIS blunting instead.

GSIS was a complicated process with several steps: the glucose transport through glucose transporter-2 (Glut2), glucose metabolism with glucokinase (GCK) involved, increased ATP/ADP ratio, membrane depolarization, and the resultant insulin granules exocytosis [Bruning et al., 1997; Lauro et al., 1998; Rorsman and Renstrom, 2003; Petersen et al., 2004]. Comprehensive effectors participated in this process including SREBP-1c, Pdx-1, and GLP-1R.

As a transcription factor, SREBP-1c was reported to regulate the expression of insulin receptor substrate (IRS)-2, Granuphilin and uncoupling protein (UPC) 2. IRS-2 was an essential signal mediator in insulin/insulin like growth factor (IGF)-1/IRS2 pathway, which was involved in the GSIS improvement [Kulkarni et al., 1999, 2002; Ueki et al., 2006]. Granuphilin, the effector of Rab27a, was specifically localized in insulin granules [Yi et al., 2002; Kasai et al., 2005] and could regulated the vesicle transport for exocytosis. Granuphilin was reported to be a direct target of SREBP-1c and its down-regulation restored the insulin release [Kato et al., 2006]. UCP2 was one member of uncoupling protein which could uncouple the energy usage and consequently decrease the ATP release in the mitochondrial inner membrane [Jacobsson et al., 1985; Bouillaud et al., 1986; Enerback et al., 1997]. Therefore, UCP2 could decrease the cytoplasmic ATP/ADP ratio [Saleh et al., 2002] and resultantly impair the GSIS [Kato et al., 2008]. Pdx-1 was the first pancreas-enriched gene product expressed in early pancreatic endocrine, exocrine, and ductal progenitors [Jonsson et al., 1994]. Recently, it was reported that specific removal of Pdx-1 in mice led to a severe diabetic phenotype [Gannon et al., 2008]. This study disclosed the

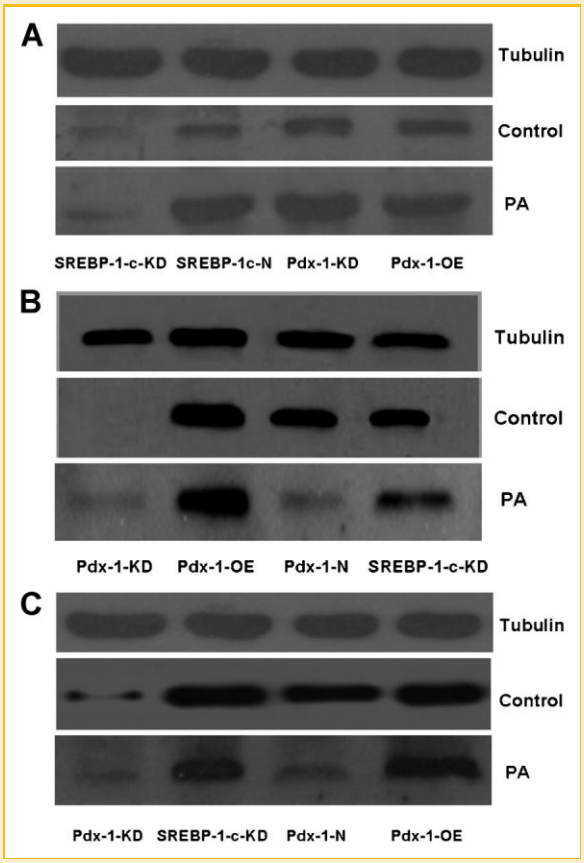

Fig. 4. Protein levels of SREBP-1c (A), Pdx-1 (B), and GLP-1R (C) in INS-1 cells, SREBP-1c-KD cells, Pdx-1-KD cells, and Pdx-1-OE cells treated without (control) or with palmitate (PA) was assayed by Western blotting. Tubulin protein was used as a loading control. Images are representative of five experiments with similar results.

importance of Pdx-1 on beta cell function. Consequently, much effort was exerted to identify the underlying mechanism in this field. Incretin, first identified by La Barre, exhibited a strong capability on correcting hyperglycemia [Creutzfeldt, 2005]. GLP-1 was a powerful incretin and could markedly enhance GSIS in beta cells through GLP-1R [Holst, 2007]. Therefore, GLP-1R was closely associated with the beta cell function.

Due to the importance of these three genes on GSIS regulation, it was necessary to investigate their expression levels in palmitate-, palmitate–EPA-, and EPA-treated INS-1 cells. The obtained data indicated that palmitate up-regulated SREBP-1c and down-regulated Pdx-1 and GLP-1R in INS-1 cells and EPA could suppress such variation. Therefore, it was speculated that the impairment and protection effects on GSIS caused by palmitate and EPA, respectively, might be attributed to, at least in part, these three genes and their targeted genes. To further identify the underlying mechanism, lentiviral system was used to knock-down SREBP-1c in INS-1 cells. It was found that the cells could normally release insulin and the GSIS was not impaired by palmitate in SREBP-1-deficient INS-1 cells (Fig. 2), indicating that the absence of SREBP-1c partially nullified palmitate-caused GSIS blunting. Furthermore, our results have demonstrated that SREBP-1c could be up-regulated by palmitate in normal INS-1 cells. Therefore, it could be considered that SREBP-1c might exert some impact in palmitate-induced lipotoxicity and the resultant GSIS impairment. Furthermore, it has been reported that chronic hyperglycemia deteriorates beta cell function with decreased Pdx-1 DNA-binding activities [Hagman et al., 2005; Kaestner and Goodman, 2007]. The effects of hyperlipemia on the expression of Pdx-1 and GLP-1R have not been reported by now. In our study, although palmitate could still suppress the expression levels of these two genes in SREBP-1c deficient cells, the extent was markedly decreased (Table I). This result demonstrated that palmitate had significantly smaller capability of down-regulating Pdx-1 and GLP-1R when SREBP-1c was absent. Based on these data, it could be speculated that SREBP-1c may act as the up-stream effector in palmitate-induced down-regulation of Pdx-1 and GLP-1R. SREBP-1c is one member of the membrane-bound transcription factor basic helix-loop-helix (bHLH) leucine zipper family. Its transcription can be induced by liver x receptor (LXR) α which is a nuclear hormone receptor and could be activated by oxysterols [Lehmann et al., 1997]. The palmitate-induced SREBP-1c up-regulation might be attributed to the activation of LXRα and further investigations are required in our further studies. IRS-2, Granuphilin, and UPC2 have been reported to be regulated by SREBP-1c. Our data further expanded the gene list and indicated that Pdx-1 and GLP-1R may be the SREBP-1c targeting genes as well. However, SREBP-1c deficiency failed to completely abolish the down-regulation effects caused by palmitate, which indicated that there might be other effectors and signal pathway involved. Further studies are required to identify this.

In addition, Pdx-1 was knocked down and over-expressed to further investigate the relationship of SREBP-1c, Pdx-1, and GLP-1R in palmitate-SREBP-1c pathway. As shown in Tables II and III, SREBP-1c displayed similar expression patterns in Pdx-1 knock-down and over-expressed cells, which demonstrated that Pdx-1 did not affect the expression of SREBP-1c. This result provided further evidence that SREBP-1c was the up-stream effector in palmitate triggered signal pathway. As for GLP-1R, its transcription was markedly suppressed when Pdx-1 was deficient. Different from the normal INS-1 cells, EPA failed to correct the palmitate-induced down-regulation of GLP-1R in Pdx-1 deficient cells. Therefore, it could be speculated that Pdx-1 was the direct upstream regulator of GLP-1R in palmitate-SREBP-1c pathway. Furthermore, in Pdx-1-OE cells, palmitate failed to decrease the expression of GLP-1R, which further demonstrated that GLP-1R may be directly regulated by Pdx-1. Pdx-1, as a transcription factor, has been found to regulate multiple genes [Kushner et al., 2002] and GLP-1R was found to be an expanded one. Taken all these findings together, GLP-1R may be the down-stream effector in palmitate triggered signal pathway. The palmitate increased the expression of SREBP-1c directly. And such increase would reduce the expression of Pdx-1, followed by the suppression of GLP-1R expression. The down-regulation of Pdx-1 by SREBP-1c occurred at the transcriptional level and its precise molecular mechanism is unclear. It was speculated that nuclear SREBP-1c might physically interact with Pdx-1 and consequently suppress the Pdx-1-autoloop activation. Additionally, there may be

other effectors and signal pathway involved in the palmitate triggered lipotoxicity pathway and further studies are required.

Insulin secretion was detected in both Pdx-1-KD and -OE cells. When Pdx-1 was knocked down, cells failed to release insulin normally with/without palmitate treatment and such GSIS impairment could not be restored by EPA. These results indicated that Pdx-1 deficiency directly resulted in the GSIS impairment (Fig. 3A). Additionally, Pdx-1-OE cells could still release insulin even though they were treated with palmitate (Fig. 3B). This finding showed that the palmitate-induced GSIS impairment was significantly nullified when Pdx-1 kept stably expressing, which further demonstrated the importance of Pdx-1. Based on the importance of GLP-1R in beta cell function, GLP-1R might act as one contributor in Pdx-1 involved GSIS improvement and further study is under investigation.

CONCLUSION

Here we showed that palmitate impaired and EPA restored insulin secretion in INS-1 cells. Additionally, palmitate could up-regulate SREBP-1c and down-regulate the expression of Pdx-1 and GLP-1R. The findings that SREBP-1c deficiency and Pdx-1 over-expression partially canceled the negative effects of palmitate on GSIS provided some evidence for the speculation that these three genes may involved in palmitate-caused GSIS impairment. Furthermore, our studies identified the relationship of SREBP-1c, Pdx-1, and GLP-1R in palmitate triggered pathway. SREBP-1c, which was up-regulated by palmitate, was the upstream effector and this was supported by the findings that palmitate failed to reduce the expression of Pdx-1 and GLP-1R in SREBP-1c deficient cells. The results that Pdx-1 knock-down/over-expression had no effect on SREBP-1c expression further verified this speculation. Moreover, the absence of Pdx-1 caused the low expression of GLP-1R. Besides, up-regulating Pdx-1 nullified the palmitate-induced down-regulation of GLP-1R. This demonstrated that it was Pdx-1 not SREBP-1c that directly regulated the expression of GLP-1R.

Taken together, the sequent SREBP-1c-Pdx-1-GLP-1R pathway contributed, at least in part, to the palmitate caused lipotoxicity in INS-1 cells. These findings would help in understanding the underlying mechanisms involved in lipotoxicity caused beta cell dysfunction.

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