

Original Article

SHIP2 on p13K/Akt pathway in palmitic acid stimulated islet β cell

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Abstract: This study is to investigate the influence of SHIP2 on palmitic acid stimulated islet β cell and insulin secretion, as well as its role in p13K/Akt pathway. We defined four groups: control, acid group, acid + NC siRNA group and acid + siRNA transfection group. The control was neither treated by palmitic acid nor transfection. The acid group was subjected to palmitic acid incubation. The acid + NC siRNA group was transiently transfected by NC siRNA, then was stimulated by palmitic acid. The acid + siRNA group was transiently transfected by siRNA, then was stimulated by palmitic acid. Cell proliferation and apoptosis were measured by MTT and flow cytometry. Immunocytochemistry, Western Blot and QPCR were designed to detect the expression of SHIP2, Akt, p-Akt protein and mRNA. Insulin secretion was tested by radioimmunoassay. The apoptosis rate in the acid + siRNA group was non-significantly lower than the acid group and the acid + NC siRNA group ($P > 0.05$). The expression levels of Akt phosphorylation in the acid + siRNA group was significantly higher than in the acid + NC siRNA group and the acid group ($P < 0.05$). And under 22.4 mmol/L glucose KRB, insulin secretion in the acid + siRNA group was significantly more than the acid + NC siRNA group and the acid group ($P < 0.05$). SHIP2 silencing probably stimulates insulin secretion, which may be associated with the enhanced proliferation in the p13K/Akt pathway.

Keywords: β TC3, p13K/Akt, phosphorylation, palmitic acid, SHIP2, RNA interference

Introduction

Type 2 diabetes mellitus (T2DM) is a worldwide disease. Insulin resistance (IR) and β -cell dysfunction are main pathogenesis of T2DM. As is known, T2DM patients, especially those obese patients, are always accompanied with a series of lipid metabolism disorders. They mainly include the elevated level of free fatty acid (FFA) and triglyceride (TG), and fat deposition in different tissues. Traditionally, IR is always used to describe the declined insulin sensitivity in insulin-sensitive tissues. Kulkarni et al., however, pointed that similar to human, mice had 85% loss of glucose-stimulated insulin secretion (GSIS), when insulin receptor gene in β cell was knockout and glucose tolerance was impaired [1]. β cell IR displays insulin signal transduction dysfunction, contributing to the reduction of insulin secretion (IS). Although this concept improved the knowledge of β cell IR, the research was still in the early stage.

At the molecular level, the activated IR leads to autophosphorylation and tyrosine phosphorylation of several substrates. Most of reactions during this process are mediated by insulin receptor substrate-1 and 2 (IRS-1 and IRS-2). Phosphorylated IRS-1 and IRS-2 activate PI3K, which gives rise to Akt serine phosphorylation. This process stimulates glucose transport, promoting fat synthesis. However, insulin signaling pathway is still unclear and needs to be researched further.

Src homology (SH2) containing inositol polyphosphate 5-phosphatase-2 (SHIP2) is a kind of protease with phosphatase activity, belonging to inositol polyphosphate 5'-phosphatase family. The latest study found that SHIP2 was able to regulate the PI3K-dependent insulin signal transduction, which played an important role in the occurrence and development of diabetes [2]. Akt, a principal downstream gene, was also regulated by PI3K. This study was

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designed to use palmitic acid as the inducing factor to establish FFA-induced β cell injury model and to investigate the effect of palmitic acid on SHIP 2 expression in β TC3 cell and IS via siRNA.

Methods

Reagent

Palmitic acid was acquired from Sigma, USA. Mouse SHIP2 monoclonal antibody (SC-166641), anti p-Akt-473 monoclonal antibody (SC-81433), anti Akt monoclonal antibody (SC-5298) were acquired from Santa Cruz Company, USA. MTT kit was acquired from Sigma, USA. Insulin radioimmunoassay kit was acquired from Purevalley Biotech Company.

Cell culture

Islet β TC3 cell line was from Denmark Novo Nordisk R & D Center. It was cultivated in RPMI medium 1640 containing 10% fetal calf serum under 37°C and 5% CO₂. After 25% cells attached, we synchronized them by the serum-free medium for 24 h. Four groups were defined: control, acid group, acid + NC siRNA group, and acid + siRNA transfection group. The control was neither treated by palmitic acid nor transfection. The acid group was subjected to palmitic acid incubation. The acid + NC siRNA group was transiently transfected by NC siRNA, then was stimulated by palmitic acid. The acid + siRNA group was transiently transfected by siRNA, then was stimulated by palmitic acid. All cells were obtained after 24 h.

Cell proliferation assay-MTT

The cells were seeded in 96-well plates and incubated with different concentrations of palmitic acid (0 mmol/L, 0.2 mmol/L, 0.4 mmol/L, 0.8 mmol/L) for 24 h, then 10 μ l MTT solution [0.5% (wt/vol) in PBS] were added for 24 h in 37°C. 120 μ l dimethyl sulfoxide (DMSO) was added into the supernatant fluid. Automated immunoanalyser was used to test optical density (A) for 3 times.

Flow cytometry

The cells were treated appropriately and then incubated for 30 min in dark at 4°C. Flow cytometry were performed (Epics-XL2 made by Beckman Coulter Company, USA) to measure

DNA content. And the data were analyzed by Expo32ADC software.

Immunocytochemistry

The cultured cells were fixed in 4% paraformaldehyde for 30 min, then were subjected to 3% H₂O₂ solution for 10 min. Briefly, mouse anti-SHIP2 (1:100) was added at 4°C, incubated overnight (PBS was used to replace primary antibodies for the control). Secondary antibody complexes were added for 20 min in 37°C. They were stained with DAB and observed by microscope.

Western blot

After the treatment of protein lysates, coomassie blue staining was used to measure protein concentration. The protein extracts (50 μ g each sample) were separated by 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was incubated overnight at 4°C with SHIP2 (1:500), Akt (1:500) and p-Akt antibody (1:500). ECL chemiluminescence was performed. β -actin was used as the reference. Protein expression levels were quantified by scanning the immunostaining band and analyzing in LabWork 45 Image Software.

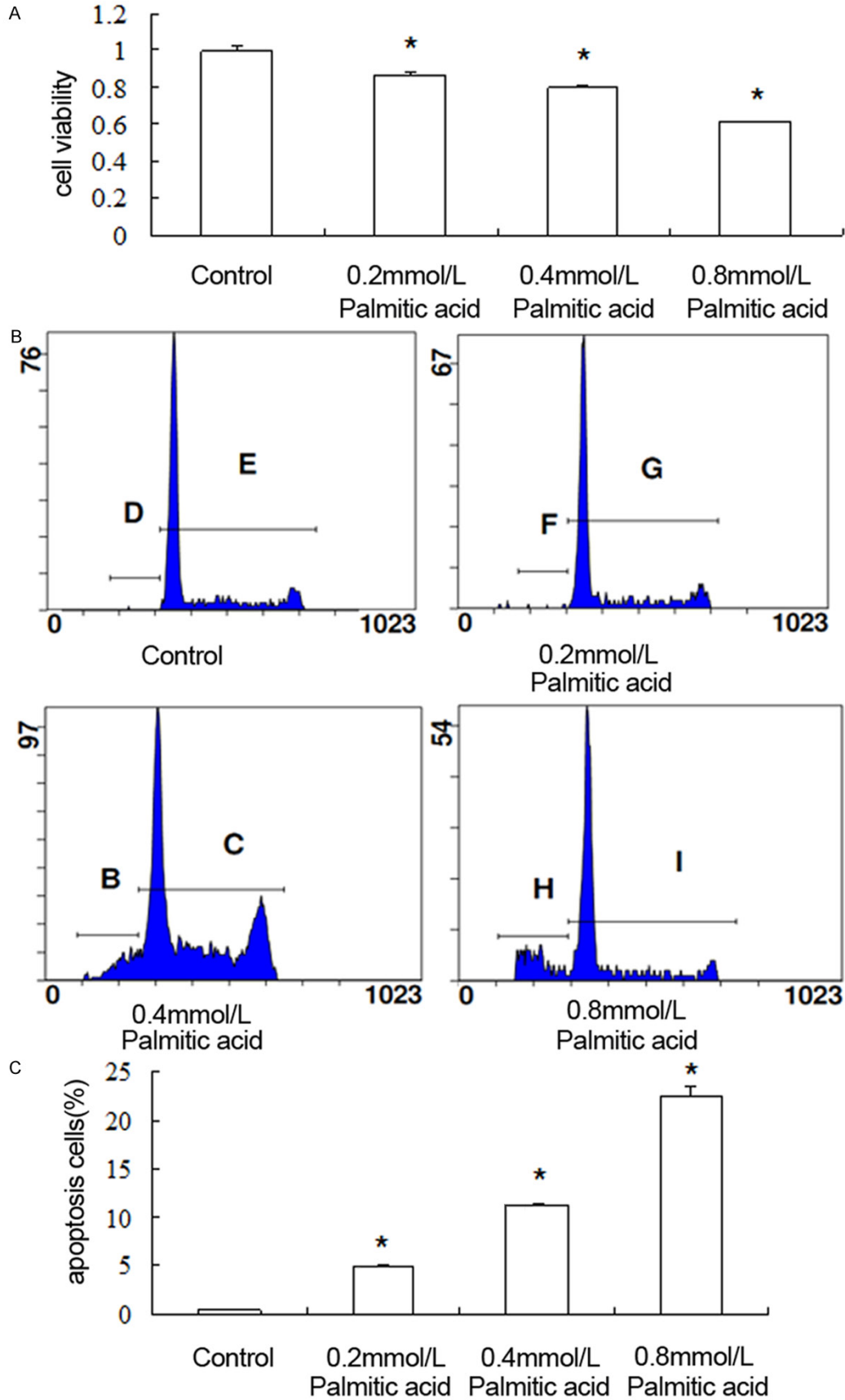
RT-PCR

We used TRIzol reagent to extract RNA. Samples were treated by reverse transcriptase to synthesize cDNA. Primers: 18S 5'-ACA CGG ACA GGA TTG ACA GA-3', 5'-GGA CAT CTA AGG GCA TCA CAG-3' (238 bp), SHIP2: 5'-ACG TGC ACA GGA AGG AGA AC-3' (583 bp). PCR cycling program was as follows: pre-incubate at 95°C for 5 minutes. Followed by 37 cycles of: Denaturation, 60 s, 94°C; Annealing, 60 s, 56°C; Extension, 60 s, 72°C. Termination 8 m, 72°C. Then, agarose gel electrophoresis was performed. The expression was analyzed by gel image analysis system (UVP Company, USA).

Radioimmunoassay

The cells were washed by KRHB buffer containing 1.6 mmol/L glucose (NaCl 119 mmol/L, KCl 4.6 mmol/L, CaCl₂ 2.5 mmol/L, MgSO₄ 1.2 mmol/L, KH₂PO₄ 1.2 mmol/L, NaHCO₃ 2.5 mmol/L, HEPES 10 mmol/L, 0.1% BSA) for 2 times. And they were incubated in containing 2.8 mmol/L and 22.4 mmol/L glucose KRHB solution respectively for 1 h in 37°C. Insulin

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Figure 1. Different concentrations of palmitic acid on (A) cell proliferation and (B) apoptosis.

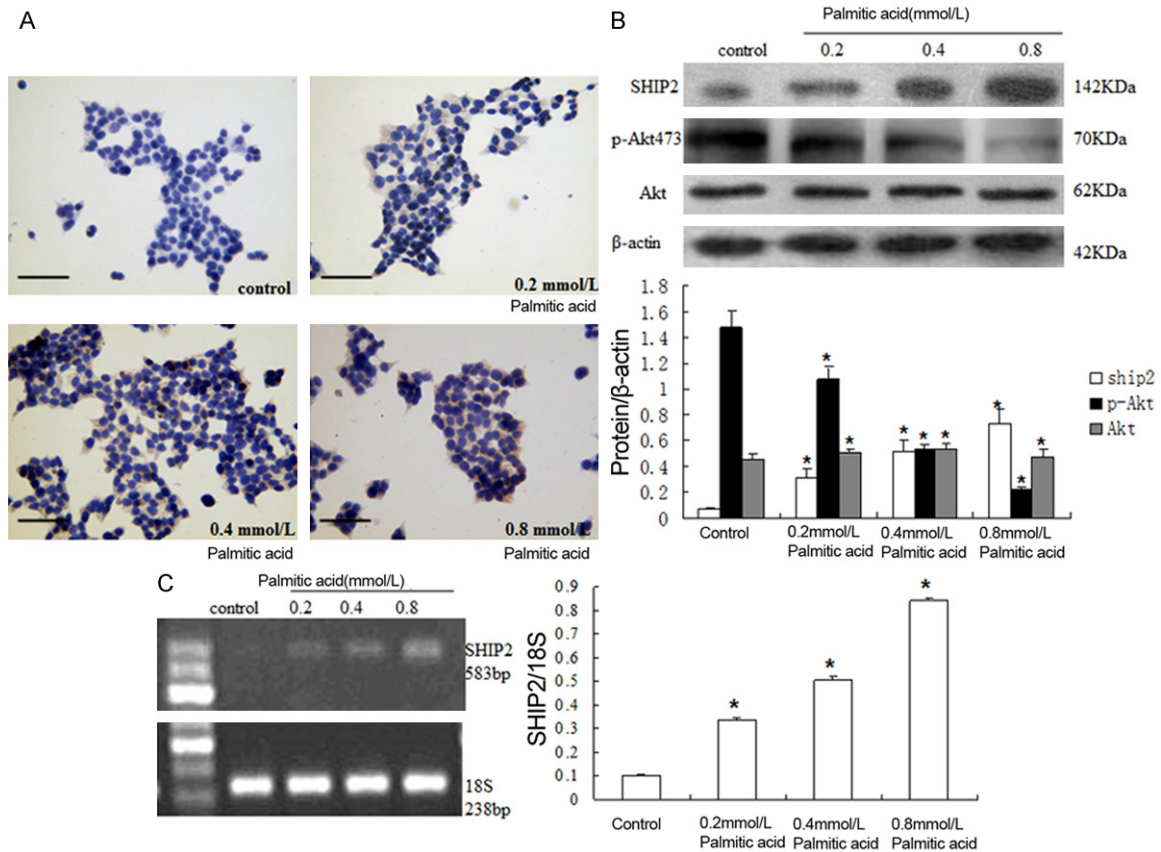


Figure 2. Different concentrations of palmitic acid on expression of SHIP2 protein and mRNA. A. Immunocytochemistry showed SHIP2 protein in cytoplasm. B. Protein expression of SHIP2, Akt and p-Akt in different concentrations of palmitic acid. C. The level of mRNA of SHIP2 in different concentrations of palmitic acid.

levels were measured by Radioimmunoassay according to the guideline of Insulin radioimmunoassay kit.

Statistics

Data are presented as means \pm S. SPSS Software V13.0 was used in the statistical analysis. The data were analyzed using one-way ANOVA with SNK test. Statistical significance was set at $P < 0.05$.

Results

Palmitic acid on cell proliferation and apoptosis

In MTT assay results (**Figure 1A**), after palmitic acid stimulation for 24 h, the β TC3 cell proliferation rate declined significantly with increasing concentrations of palmitic acid, indicating

that palmitic acid dose-dependent inhibited proliferation. In flow cytometry results (**Figure 1B**), The apoptosis rate of the control was $(3.03 \pm 0.03)\%$. And the β TC3 cell apoptosis rates were $(4.97 \pm 0.14)\%$, $(11.07 \pm 0.48)\%$, $(22.43 \pm 1.01)\%$ in 0.2 mmol/L, 0.4 mmol/L, 0.8 mmol/L palmitic acid for 24 h respectively. The apoptosis rate was positively associated with concentrations of palmitic acid ($P < 0.05$).

Palmitic acid on expression of SHIP2 protein and mRNA

In results of immunocytochemistry (**Figure 2A**), SHIP2 protein represented brown grains in cytoplasm and distributed uniformly. With the increase of palmitic acid concentration, the expression of SHIP2 in cytoplasm enhanced. In Western blot results (**Figure 2B**), compared to the control, the expression of SHIP2 protein

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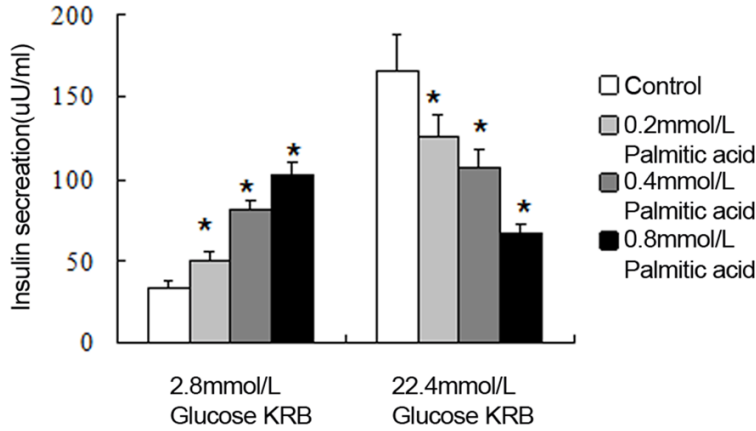


Figure 3. Insulin secretion in different concentrations of palmitic acid and glucose KRB.

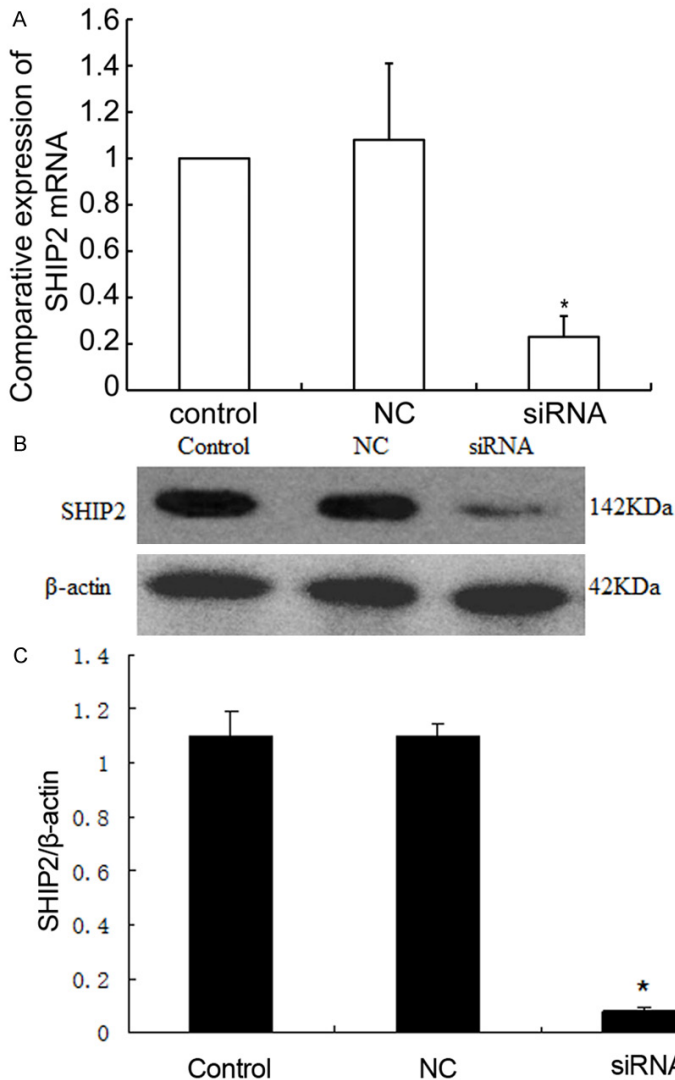


Figure 4. (A) SHIP2 gene and (B) SHIP2 protein expression after siRNA intervention.

was higher after 24 h palmitic acid incubation and enhanced with the increase of concentration. As is shown in RT-PCR (**Figure 2C**), compared to the control, the level of mRNA of SHIP2 rose with the increase of concentration, indicating that palmitic acid probably induced SHIP2 mRNA expression. And its concentration was positively associated with the expression level.

Palmitic acid on Akt phosphorylation level

As is shown in **Figure 2B**, there was no significant difference among groups on the Akt expression. Compared to the control, p-Akt level in βTC3 cells considerably declined with the increase of concentration.

Palmitic acid on insulin secretion

In radioimmunoassay (**Figure 3**), after 24 h palmitic acid incubation, insulin secretion increased gradually with increasing concentration of palmitic acid under 2.8 mmol/L glucose KRB. Under 22.4 mmol/L glucose KRB, however, insulin secretion was negatively associated with concentration of palmitic acid.

siRNA intervention

After 48 h transient transfection, the expressions of samples were measured by qPCR (**Figure 4A**). And in Western blot results, the SHIP2 protein expression in the siRNA group was slower than the control and the NC siRNA group. And there was no significant difference between the control and the NC siRNA group ($P = 0.6099$, **Figure 4B**).

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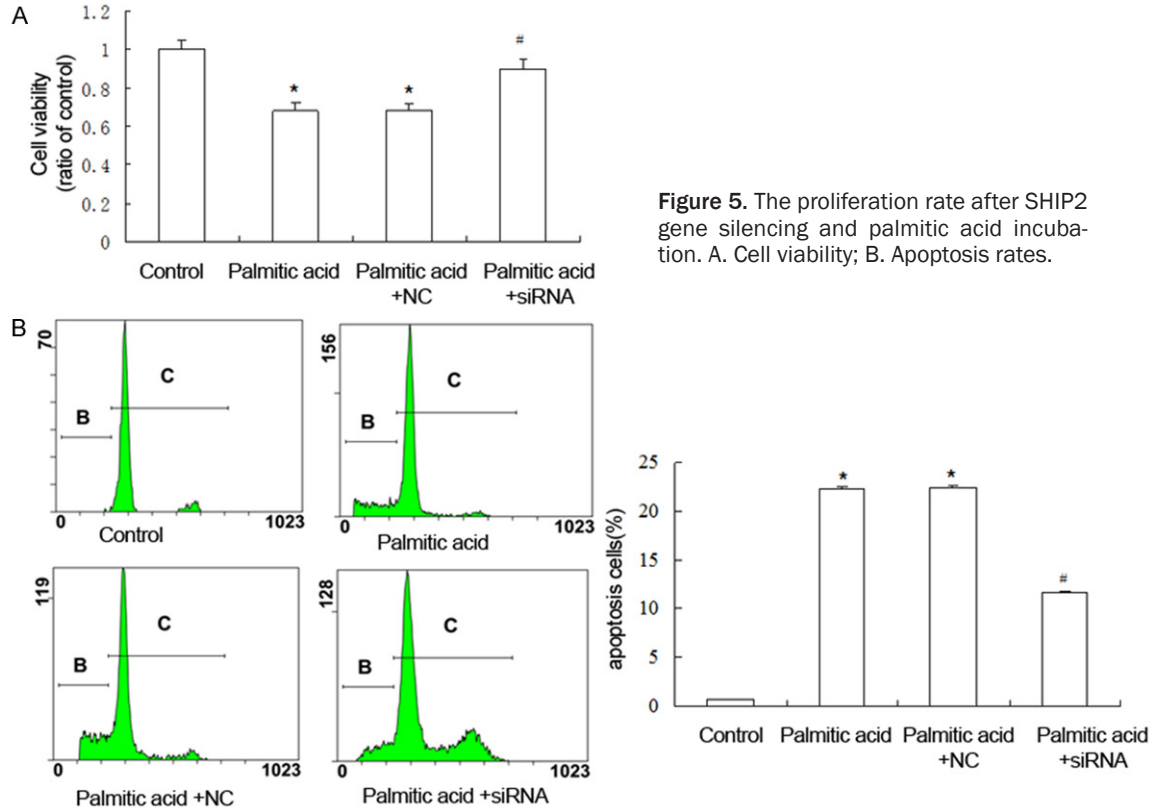


Figure 5. The proliferation rate after SHIP2 gene silencing and palmitic acid incubation. A. Cell viability; B. Apoptosis rates.

SHIP2 gene silencing on proliferation and apoptosis

SHIP2 gene silencing significantly promoted the β TC3 cells proliferation. In **Figure 5**, the proliferation rate in the acid + siRNA group was significantly higher than the acid + NC siRNA group and the acid group at 48 h. Moreover, the apoptosis rate in the acid + siRNA group was $(3.89 \pm 0.023)\%$, which was non-significantly lower than the acid group $[(3.91 \pm 0.021)\%]$ and the acid + NC siRNA group $[(3.98 \pm 0.025)\%]$ ($P > 0.05$).

SHIP2 gene silencing up-regulated p-Akt level

As for the expression of Akt phosphorylation in three groups, the level in the acid + siRNA group (0.572 ± 0.035) was significantly higher than in the acid + NC siRNA group (0.145 ± 0.011) and the acid group (0.143 ± 0.015) (**Figure 6**).

SHIP2 gene silencing increased insulin secretion

There was no significant difference among three groups on insulin secretion under 2.8 mmol/L glucose KRB. And under 22.4 mmol/L

glucose KRB, insulin secretion in the acid + siRNA group was significantly more than the acid + NC siRNA group and the acid group ($P < 0.05$) (**Figure 7**).

Discussion

T2DM is a systemic metabolic disorder syndrome, and derived from the lack of insulin secretion and IR. So far, its mechanism is still unclear, but most studies pointed that apoptosis of islet β cells was associated with the development of T2DM [1, 2]. Epidemiological surveys showed that T2DM was closely associated with fat [3].

For the obese patients, especially abdominal obesity, FFA level in plasma is always abnormally high [4]. At the early stage of T2DM, elevated FFA induces mild degree of IR. However, islet β cells secrete more insulin to stabilize blood glucose via compensatory hyperplasia [5]. Decompensation damages the function of islet β cells and inhibits the proliferation of normal cells. So, lack of insulin makes the blood glucose out of control, leading to diabetes finally [6]. Therefore, FFA plays a major role in the

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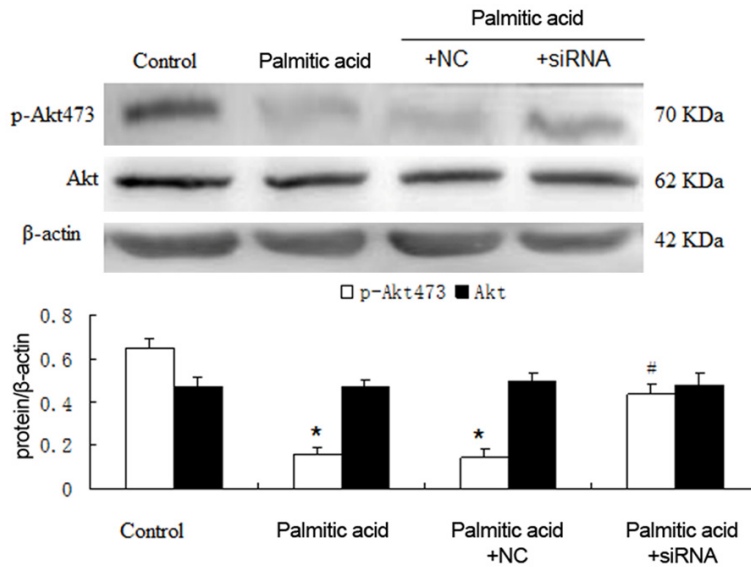


Figure 6. p-Akt levels after SHIP2 gene silencing and palmitic acid.

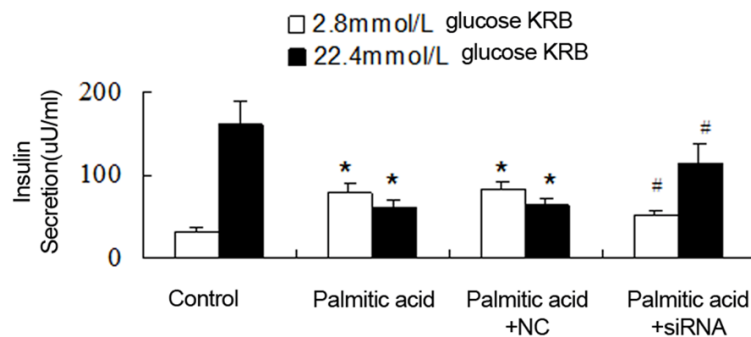


Figure 7. Insulin secretion after SHIP2 gene silencing and palmitic acid.

proliferation and apoptosis of pancreatic β cells.

Palmitic acid, a saturated fatty acid, is able to decrease the proliferation of pancreatic β cells and induce apoptosis. But, 9-hexadecenoic acid, a monounsaturated fatty acid, can neutralize the toxic effect of palmitic acid and promote proliferation of β cell. As is researched, palmitic acid derived apoptosis can activate cytochrome C to release caspase-3 [7]. Stimulated by palmitic acid, GSIS is damaged [8, 9]. More importantly, excess fatty acid is able to induce pancreatic β cell apoptosis directly [10, 11]. In this study, we investigated the β TC3 cells exposed in different concentrations of palmitic acid for 24 h. And the results showed declined proliferation, increased apoptosis, and reduced IS level in the pancreatic β cells. So, it indicated that palmitic acid induced

apoptosis was possibly one of the main reasons inhibiting β cell growth and IS, which was consistent with the results of Maedler et al [12].

Akt is a major downstream target of PI3K pathway. Recent studies indicated that PI3K/Akt signaling pathway was involved in islet β cell cycle progression and survival process. Further, PI3K has been proved to be a required condition for the insulin signaling pathway. And insulin maintains blood glucose levels and balance of glycogen synthesis and gluconeogenesis via PI3K signaling pathway. And PI3K, one of important lipid kinase, regulates insulin sensitivity via Akt. After binding to the insulin receptor, insulin promotes phosphatidylinositol 3,4-bisphosphate (PIP₂) to generate Phosphatidylinositol 3,4,5-triphosphate (PIP₃) via activation of PI3K. Our results revealed that hexadecanoic acid prompted p-Akt expression in β TC3 cells. SHIP2 gene, an inositol 5'-phosphatase gene highly homologous with SHIP1, was found in recent

years. Contrast to SHIP1, SHIP 2 was not only expressed in hematopoietic cells, but in many kinds of tissue cells [13]. SHIP2 was also demonstrated to be dependent on insulin signal transduction pathway. Overexpression of SHIP2 reduced activation of insulin-stimulated MAP kinase and Akt, and declined glucose transport [8]. In SHIP2 gene knockout mice, high insulin sensitivity increased the second messengers and activated Akt, leading to a series of biological effects further. As the suppressor in insulin signal transduction pathway, SHIP2 hydrolyzed PIP₃ via 5'-phosphatase activity, and inhibited Akt phosphorylation to regulate blood glucose level [14]. And recent researches showed that PIP₃ level was considerably higher in SHIP2 $-/-$ mice than SHIP2 $+/+$ mice, which was contrast with PI(3, 4)P₂ [15]. So, SHIPs probably effected growth factors and insulin via PI3K signaling pathway to regulate IS and IR [16, 17].

In this study, we investigated the effect of palmitic acid on p-Akt and SHIP2 in β TC3 cells. And the results revealed that palmitic acid down-regulated p-Akt, up-regulated protein and mRNA of SHIP2. And all the reactions were dose-dependent. We also performed RNA interference to treat the β TC3 cells, and designed 3 strands of Stealth siRNA. The positive-sense strand was subjected to chemical modification to eliminate the RNA interference of positive-sense strand and non-specific inhibition. The effect of Stealth siRNA1-3 on SHIP2 was examined by PCR. According to the silence efficiency, we chose Stealth siRNA2 to silence SHIP2 in β TC3 cells. The insulin secretion in the acid + siRNA group was significantly more than the other groups, which was possibly associated with the proliferative activity. The MMT analysis further confirmed this result. The promotion of proliferative was top from 48 h to 72 h of transfection, which was shown in proliferation curves. Flow cytometry revealed that the apoptosis rate in the acid + siRNA group was lower than the acid group and the acid + NC siRNA group ($P > 0.05$). Moreover, after SHIP2 silencing, the significantly elevated Akt phosphorylation level and declined IS indicated that SHIP2 reduced islet β cell proliferation and IS via inhibit of Akt phosphorylation and Akt downstream target genes.

In conclusion, SHIP2 gene expression loss activated PI3K/Akt signaling pathway, which played an important role in IS of islet β cell and was probably a novel target for the treatment of type 2 diabetes.

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Disclosure of conflict of interest

None.

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