Neutral ceramidase activity inhibition is involved in palmitate-induced apoptosis in INS-1 cells

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Abstract. Neutral ceramidase (NCDase) is a class of ceramidases, a key enzyme in ceramide degradation. Recently, it was observed that NCDase activity was suppressed by saturated fatty acids to increase ceramide content in rat muscle. However, little is known about its changes in activity and roles in palmitate (Palm)-induced lipotoxicity in pancreatic β cells. Here, we demonstrated that Palm treatment significantly down-regulated NCDase activity, mRNA and protein levels in rat INS-1 cells. In addition, Palm caused a significant accumulation of ceramide, while SPH level remained unchanged, suggesting that inhibition of NCDase activity led to no change of SPH level after treatment with Palm for 24 h. Furthermore, NCDase overexpression significantly reduced Palm-induced apoptosis in INS-1 cells. Conversely, NCDase siRNA knockdown markedly exacerbated Palm-induced apoptosis. In conclusion, Palm treatment suppressed the activity of NCDase and down-regulated its mRNA and protein expression. Furthermore, NCDase inhibition was involved in Palm-induced apoptosis by blocking ceramide degradation in INS-1 cells.

Key words: Neutral ceramidase, Palmitate, Apoptosis, Ceramide, Pancreatic β-cells

TYPE 2 DIABETES MELLITUS is characterized by insulin resistance and impaired pancreatic β-cell function. Deleterious effects of excessive fatty acid deposition in non-adipose tissues (such as islets and pancreatic β cells), a phenomenon known as lipotoxicity [1], are involved in the pathogenesis of β-cell damage. Elevated levels of circulating free fatty acids (FFAs) are commonly found in patients with type 2 diabetes [2]. More importantly, excess FFAs are implicated in the inhibition of pancreatic β-cell insulin secretory responsiveness and the induction of β-cell apoptosis [3]. Furthermore, the excess of saturated FFAs, such as palmitate (Palm), which induces lipotoxicity in primary human and mouse hepatocytes, have been implicated in insulin resistance in liver and muscle cells [4, 5].

Accumulating evidence reveals that Palm-induced β-cell apoptosis is associated with ceramide accumulation in pancreatic islets [6, 7]. Ceramide is a bioactive sphingolipid, involved in the cellular processes associated with apoptosis, cell growth arrest, senescence, cell migration and adhesion [8-10]. Ceramide can be produced in at least two distinct ways. Firstly, endogenous ceramide can be generated via the de novo pathway, in which serine and palmitoyl CoA condense to form 3-ketosphinganine by serine palmitoyl transferase (SPT), followed by reduction of 3-ketosphinganine to sphinganine, which is then N-acylated by ceramide synthases to produce dihydroceramide, followed by desaturation of dihydroceramide desaturase to ceramide [11]. Secondly, the generation of ceramide is triggered by the action of sphingomyelinases (SMases) which hydrolyze sphingomyelin (SM) to yield ceramide [11]. In the animal and human models, Palm exposure caused a significant increase in total ceramide content by activating SPT and enhancing ceramide synthesis de novo, resulting in β-cells apoptosis and insulin secretion inhibition.
[7], whereas blockage of de novo ceramide synthesis prevented Palm-induced inhibition of insulin expression [12]. Therefore, Palm induced β-cell dysfunction and endogenous ceramide accumulation through promoting ceramide synthesis de novo.

Ceramide can be utilized as a substrate by ceramidases (CDases) to liberate sphingosine (SPH). CDases are categorized as acidic, neutral or alkaline based on their pH optimum and subcellular localization [13]. Several studies indicate that neutral ceramidase (NCDase) plays pivotal roles in promoting cell proliferation and preventing cell death [14, 15]. Our previous work indicated that rat insulin-secreting INS-1 cells possessed the ability to synthesize and secrete NCDase and NCDase protected INS-1 cells against cytokine-induced apoptosis by promoting ceramide degradation [16, 17]. However, the effect of NCDase on Palm-induced β cell apoptosis is not fully investigated. In the present study, we investigated the changes in NCDase activity and protein expression and their roles in Palm-induced lipotoxicity in rat insulin-secreting INS-1 cell line.

**Materials and Methods**

**Cells culture and reagents**

Rat insulinoma INS-1 cells were maintained in RPMI-1640 medium containing 11 mM glucose, 2 mM L-glutamine, 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 units/mL penicillin and 100 μg/mL streptomycin. INS-1 cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Culture medium and supplements were purchased from Gibco (Carlsbad, CA, USA). Palm was obtained from Sigma-Aldrich (St. Louis, MO). Fatty acid-free bovine serum albumin (BSA-V) was purchased from Roche (Basel, Switzerland). The BCA protein assay kit was obtained from KeyGen BioTech (Nanjing, China). The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). C17-D-erythro-SPH and C12-ceramide were purchased from Avanti Polar Lipids (Alabaster, AL, USA). C12-D-erythro-SPH was obtained from Larodan (Malmö, Sweden).

**Palm preparation**

Palm solution was prepared as described previously [18]. Briefly, a 100-mM Palm stock solution was prepared in 0.1 M NaOH in a 70°C shaking water bath. Five percent (wt/vol) FFA-free BSA solution was formulated in double-distilled H₂O. A 5-mM Palm/BSA solution was obtained by conjugating both Palm and 5% BSA in a particular ratio in a 60°C water bath. The above solution was then cooled to room temperature, filtered and stocked at -20°C. The desired concentration was prepared by combining the Palm/BSA solution and complete medium in the appropriate proportion and was administered to cultured cells.

**MTT assay**

1×10⁴ cells per well were seeded in 96-well plate for 24 h. Then cells were cultured with Palm (0.25 mM) and Palm (0.5 mM) for different time periods (4, 8, 12, 16 and 24 h). And BSA treatment was considered as control group. Before removing Palm treatment, 5 g/L MTT solution was added into the wells for a final concentration of 0.5 g/L. After incubated for 4 h at 37°C in an atmosphere containing 95% air and 5% CO₂, the MTT solution was removed, 150 µL Dimethyl sulfoxide solution was added, and then the absorbance at 570 nm was measured with microplate reader.

**Real-time PCR analysis**

Total RNA was extracted using Trizol lysis reagent (Invitrogen, USA) after INS-1 cells were treated with Palm (0.5 mM) for different time periods (4, 8, 12, 16 and 24 h). And BSA treatment was considered as control group. Before removing Palm treatment, 5 g/L MTT solution was added into the wells for a final concentration of 0.5 g/L. After incubated for 4 h at 37°C in an atmosphere containing 95% air and 5% CO₂, the MTT solution was removed, 150 µL Dimethyl sulfoxide solution was added, and then the absorbance at 570 nm was measured with microplate reader.

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**Flow cytometry apoptosis analysis**

Cells were collected with 0.25% trypsin-EDTA, rinsed with pre-cooled PBS, and then resuspended in binding buffer. Apoptosis was analyzed using an annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions. The cells were then incubated for 15 min at room temperature in the dark. Subsequently, they were detected with the Guava EasyCyte flow cytometer (Millipore, Boston, USA) and analyzed by Guavasoft.

**Western blot analysis**

Following Palm treatment, the cells were harvested by trypsinization, washed with PBS and lysed in RIPA buffer (Beyotime Biotechnology, P0013E-1) supplemented with 1 mM PMSF, a protease inhibitor. Protein concentration was determined using the BCA assay. Total protein extracts (40 μg) were separated on a 10% SDS-PAGE gel and then electrotransferred to PVDF membranes. β-actin was used as a loading control for all samples. Blots were blocked with 5% nonfat dried milk and then incubated with anti-β-actin (Cell Signaling Technology, 1:5,000) and anti-NCDase (ThermoFisher Scientific, ab63804, 1:10,000) primary antibodies at 4°C overnight. Blots were subsequently incubated with the corresponding peroxidase-conjugated secondary antibodies (Cell Signaling Technology, USA) at room temperature. Bands were detected with an ECL western blotting detection kit (Millipore, USA) and band intensities were quantified using Quantity One software.

**NCDase activity assay**

NCDase activity was detected by determining the amount of sphingoid base released from ceramides using an HPLC assay, as described previously [16]. Briefly, the supernatant was collected and protein concentration was determined by the BCA method. C12-ceramide was dissolved in 0.2M HEPES buffer (pH 7.5, 0.2% Triton X-100, 5 mM CaCl2) and for a final concentration of 100 μM. C12-ceramide as a substrate was added in each 100-μg sample for 2 h at 37°C. HPLC-grade methanol and chloroform were used to stop and dissolve reaction products. C17-D-erythro-SPH (50 pmol) was then added for 2 h at 37°C shaking water bath. After the samples were cooled, chloroform and NaCl-5% glycerinum were added as extraction solvents to each sample. Fresh o-phthalaldehyde was then added to each sample. The o-phthalaldehyde derivative of released SPH was determined by HPLC with C12-D-erythro-SPH as an external standard and C17-D-erythro-SPH as an internal standard.

**INS-1 cell transfection with recombinant plasmid pEGFP-C3-NCDase**

The empty vector (pEGFP-C3) was purchased from Clontech, and the recombinant plasmid (pEGFP-C3-NCDase) was a gift from Prof. Hannun (Medical University of South Carolina, USA). The INS-1 cells were transfected with recombinant plasmid (pEGFP-C3-NCDase) using Lipofectamine™ 2000 in accordance with the manufacturer’s instructions (Invitrogen, Life Technologies) and then inoculated into new cell culture plates. We selected for transformed clones using 400 μg/mL G418 following transfection for 24 h. G418-containing medium was replaced every 3–4 days. After 2 weeks of selection, medium containing 200 μg/L G418 was used for selection until colonies were visible. Monoclonal cells were then inoculated into cell culture flasks to continue culture.

**INS-1 cell transfection with NCDase siRNA**

The INS-1 cells were plated onto a 6-well plate (3×10^5 cells/well) and grown overnight to 60–70% confluence and cells were then transfected with 20 nM NCDase-targeted or scrambled (silencer negative control #1) siRNA oligonucleotides (both from ThermoFisher Scientific, MA, USA) that were complexed with Lipofectamine™ 2000 in serum-free Opti-MEM medium according to the manufacturer’s instructions. After 24 h incubation with siRNA-lipofectamine complexes, growth medium was replaced with RPMI-1640 medium supplemented with 0.5 mM Palm for 24 h.

**Intracellular sphingolipids Measurements**

Following NCDase overexpression or siRNA knockdown treatment, INS-1 cells were incubated with Palm for 24 h and then were washed three times with NaCl/ Pi before total lipids were extracted using the BlighDyer method. The extracted lipid aliquot was saved for total phosphate determination. Electrospray ionization tandem mass spectrometry was used for quantitative determination of sphingolipids (ceramide and SPH) as described previously [17].

**Statistical analysis**

All values are expressed as mean ± SD from at least three independent experiments. Significance was
tested by Student’s t-test or one-way ANOVA followed by Newman–Keuls test. *p < 0.05 was considered statistically significant.

**Results**

**The effect of Palm on INS-1 cell viability**

To detect the effect of Palm on the viability of INS-1 cells, cells were treated with Palm at various concentrations (0.25 and 0.5 mM) for the indicated time periods. Cell viability was determined by MTT assay. As shown in Fig. 1, the half maximal (50%) inhibitory concentration (IC50) was 0.5 mM at 24 h in INS-1 cells.

**Palm inhibited NCDase activity**

To determine whether NCDase activity was affected by Palm, cells were treated with Palm (0.5 mM) for the indicated time points. NCDase activity was then analyzed by quantification of the released sphingoid base from ceramide using an HPLC assay. Our results demonstrated that NCDase is active in INS-1 cells (20.12 ± 1.51 pmol SPH/min/mg in the control cells). However, Palm exposure led to a time-dependent inhibition of NCDase activity. NCDase activity began to decline markedly at 12 h after treatment with Palm (72.93 ± 5.35% of that of the control, *p < 0.05) and remained lower after 16 h and 24 h (55.76 ± 4.38% of that of the control, **p < 0.01; Fig. 2).

**Palm down-regulated NCDase mRNA levels and protein expression**

To investigate the effect of Palm on NCDase mRNA and protein expression in INS-1 cells, cells were incubated with Palm for the indicated time points. NCDase mRNA and protein levels were detected by real-time PCR analysis and western blot assay, respectively. We found that NCDase mRNA and protein levels began to reduce significantly at 12 h after incubation with Palm (*p < 0.01) and continued to decline before reaching a plateau at 24 h (Fig. 3).

**NCDase overexpression alleviated Palm-induced apoptosis in INS-1 cells**

To explore the effect of NCDase overexpression on Palm-induced apoptosis, INS-1 cells transfected with pEGFP-C3-NCDase and pEGFP-C3-vector were treated with Palm or BSA control medium for 24 h. the proportions of apoptotic cells were determined using the Annexin V-FITC apoptosis detection kit by flow cytometry. As shown in Fig. 4A, the cells were transfected successfully with pEGFP-C3-NCDase, and NCDase protein was overexpressed. Apoptosis was significantly induced in the pEGFP-C3 plus Palm group, compared with that in the pEGFP-C3 plus BSA group (28.73 ± 1.76% vs. 7.26 ± 0.68%; Fig. 4B). Interestingly, we observed that the proportion of apoptotic cells was markedly reduced in the

![Fig. 1](image1.png)  **Fig. 1** Effects of Palm on cell viability in INS-1 cells

Cells were treated with Palm 0.25 mM (grey bar) and Palm 0.5 mM (black bar) for the indicated time points and cell viability was measured by MTT assay. $^S p<0.05$ vs the BSA group of the Palm (0.25 mM); * $p<0.05$, ** $p<0.01$ vs the BSA group of the Palm (0.5 mM).

![Fig. 2](image2.png)  **Fig. 2** Effects of Palm (0.5 mM) on NCDase activity in INS-1 cells

Cells were treated with Palm for the indicated time periods, and NCDase activity was measured by HPLC assay. * $p<0.05$ vs the BSA group.
Fig. 3  Effects of Palm (0.5 mM) on NCDase mRNA and protein expression in INS-1 cells

Cells were exposed to Palm for different time periods. (A) The mRNA levels were detected by real-time PCR. (B) NCDase protein expression was determined by western blotting analysis and band intensities were quantified by the Quantity One software. The values are expressed as a percentage of the control after normalization to β-actin. * p<0.05, ** p<0.01 vs the BSA group.

Fig. 4  Effect of NCDase overexpression on Palm-induced apoptosis in INS-1 cells

(A) NCDase overexpression was established in INS-1 cells by transfection with the recombinant plasmid pEGFP-C3-NCDase. (B) Apoptotic cells were determined by flow cytometry after treatment with Palm for 24 h. BSA (light grey bar) and Palm (dark grey bar), ** p<0.05 vs pEGFP-C3 plus BSA, *** p<0.01 vs pEGFP-C3 plus Palm (n = 3).
pEGFP-C3-NCDase plus Palm group compared with that in the pEGFP-C3 plus Palm group (14.24 ± 1.25% vs. 28.73 ± 1.76%; Fig. 4B).

**NCDase siRNA exacerbated Palm-induced apoptosis**

To examine whether NCDase siRNA exacerbated Palm-induced apoptosis, cells were transfected with control siRNA (con. siRNA) or NCDase siRNA before treatment with Palm or BSA control medium for 24 h. NCDase protein expression was significantly inhibited in the NCDase siRNA group compared with that in the con. siRNA group (Fig. 5A). Although treatment of INS-1 cells with NCDase siRNA did not alone induced remarkable apoptosis, NCDase siRNA co-treatment with Palm remarkably exacerbated Palm-induced apoptosis in the con. siRNA group (39.64 ± 1.02% vs. 27.97 ± 1.22%; Fig. 5B).

**Ceramide accumulation partly resulted from Palm-induced NCDase inhibition**

To determine whether ceramide accumulation was partly owing to Palm-induced NCDase inhibition, NCDase overexpression and siRNA knockdown were performed in INS-1 cells before treatment with Palm or BSA control medium for 24 h. Intracellular ceramide and SPH levels were measured by electrospray ionization tandem mass spectrometry. Our data showed that Palm significantly induced an increase

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**Fig. 5** Effect of NCDase siRNA knockdown on Palm-induced apoptosis in INS-1 cells

(A) INS-1 cells were transfected with NCDase siRNA or scrambled control siRNA, and western blotting was performed to determine NCDase protein expression. (B) Apoptotic cells were detected by flow cytometry after treatment with Palm for 24 h. BSA (light grey bar) and Palm (dark grey bar), **p<0.05 vs con. siRNA plus BSA, $$$ p<0.01 vs con. siRNA plus Palm (n = 3).**
in ceramide content but not SPH, and the level of SPH remained unchanged in Palm group, compared with the BSA control group. As expected, pEGFP-C3-NCDase plus Palm markedly reduced intracellular ceramide content and increased SPH level, compared with the Palm group (p<0.05), whereas NCDase siRNA plus Palm significantly exacerbated Palm-stimulated ceramide accumulation and reduced intracellular SPH level, compared with the Palm group (p<0.05; Fig. 6).

**Discussion**

NCDase is a key enzyme involving in ceramide degradation. Our previous work showed that NCDase can be produced and secreted from rat insulinoma INS-1 cells and NCDase was involved in protection against cytokine-induced toxicity in INS-1 cells. Palm is a precursor of palmitoyl CoA [19], which is implicated in ceramide synthesis de novo. Accordingly, excessive Palm induced ceramide accumulation via promoting ceramide synthesis de novo and caused β cell impairment [20]. Interestingly, an in vivo animal study revealed that inhibition of NCDase activity was an underlying mechanism by which saturated fatty acid induced endogenous ceramide accumulation in rat skeletal muscle [21]. To date, the effect of Palm on NCDase activity has not been investigated in pancreatic β cells.

In the present work, we demonstrated for the first time that NCDase activity was inhibited by Palm in the INS-1 pancreatic β cell line, a known in vitro model currently used for the study of pancreatic β-cell function and apoptosis. NCDase activity from camel brain can be significantly inhibited by phosphatidylserine at low concentration [22]. Phosphatidylserine was generated from the metabolic interaction between glucose and Palm in beta cells [23]. Therefore, we speculate that Palm-induced inhibition of NCDase activity may

![Fig. 6](image-url)  
**Fig. 6** Effects of NCDase on the Palm-induced increase in ceramide content in INS-1 cells  
(A, C) Stable pEGFP-C3 and pEGFP-C3-NCDase transformants of INS-1 cells overexpressing NCDase were treated with Palm (dark grey bar) or BSA control medium (light grey bar) for 24 h. Ceramide levels were detected by electrospray ionization tandem mass spectrometry. *p<0.05, **p<0.01 vs the BSA control group; #p<0.01 vs Palm group; ns: no significance (n=3).  
(B, D) Cells were transfected with control siRNA (con siRNA) or NCDase siRNA before treatment with Palm (dark grey bar) or BSA control complete medium (light grey bar) for 24 h. Intracellular ceramide content and SPH level were measured by electrospray ionization tandem mass spectrometry. *p<0.05, **p<0.01 vs the BSA control group; §§ p<0.01 vs Palm group; ns: no significance (n=3).
be associated with generation of mentioned lipid messengers. Similarly, we found that NCDase mRNA and protein levels were down-regulated in response to Palm in a time-dependent manner. Thus, further research is needed to clarify the potential mechanism by which Palm induces NCDase inhibition in pancreatic β cells.

Furthermore, our findings indicated that NCDase overexpression significantly reduced Palm-induced β-cell apoptosis, while NCDase siRNA enhanced Palm-induced apoptosis, maybe resulting from more inhibition of NCDase protein and activity in NCDase siRNA plus Palm group, compared to that of NCDase siRNA plus BSA group (Supplemental Fig. 1). These data show that NCDase plays a pivotal role in protection against Palm induced β-cell apoptosis. Growing evidence has demonstrated that ceramide is a pro-apoptotic mediator involved in Palm-induced apoptosis [24, 25]. Ceramide was implicated in growth arrest and apoptosis [25] and as a substrate, can be catalyzed by NCDase to produce SPH. Interestingly, in the present study, non-obvious change in SPH level was observed after Palm treatment either in control cells or in pEGFP-C3-NCDase cells, although Palm caused a marked increase in ceramide. The reasons why increased ceramide was not accompanied by an increase in SPH after Palm treatment might be as follows: First, NCDase plays an important role in catalyzing conversion of ceramide to SPH, whereas we found that Palm treatment resulted in inhibition of NCDase protein and activity, which may hinder the generation of SPH from ceramide hydrolysis. Second, SPH can be acylated to ceramide via salvage synthesis pathway. It was reported that exposure of mouse pancreatic beta-cell to Palm induced the level of ceramide synthase, the enzyme of the ceramide salvage pathway, which promoted the conversion of SPH to ceramide [26]. Therefore, we speculate that inhibition of NCDase activity and stimulation of SPH converting to ceramide after Palm treatment might lead to non-obvious increase in SPH, inconsistent with the increase in ceramide. Additionally, Palm failed to markedly change the level of exogenous overexpressed NCDase (pEGFP-NCDase) driven by the CMV promoter, compared to that of BSA group in pEGFP-C3-NCDase cells (Supplemental Fig. 2A), this data suggested that Palm has no effect on exogenous pEGFP-NCDase protein expression but just inhibited endogenous NCDase expression. Anyway, activity of NCDase was elevated significantly in pEGFP-C3-NCDase cells (Supplemental Fig. 2B). And our work indicated that overexpression of NCDase significantly alleviated ceramide accumulation induced by Palm and increased SPH level, compared to that of control cells treated with Palm. As shown in Supplemental Fig. 1, NCDase knockdown inhibited markedly protein and activity of NCDase and Palm aggravated the inhibition of protein and activity of NCDase in NCDase siRNA cells. Furthermore, we found that NCDase knockdown enhanced Palm-induced ceramide accumulation and caused a significant decrease in SPH level. Take together, our data suggest that NCDase inhibition is involved in Palm-induced apoptosis in INS-1 cells via blocking ceramide degradation and NCDase inhibition may be a novel pathway for ceramide accumulation, in addition to the ceramide synthesis de novo. Previously, we had reported that INS-1 cells exhibited basal NCDase activity [16], basal acid CDase activity was also detected in INS-1 cells and isolated rat islets [27]. In current study, we found that Palm treatment reduced mRNA and protein levels of NCDase, suppressing its activity and accumulating ceramide. The effect of Palm on NCDase in INS-1 cells looks like enzyme specific because it has no significant effect on acid or alkaline CDases (data not shown).

In summary, the present study demonstrated that Palm treatment inhibited NCDase activity, mRNA and NCDase protein expression in INS-1 cells and revealed that NCDase inhibition was involved in Palm-induced apoptosis of pancreatic β cells via reducing ceramide degradation. More importantly, NCDase can be secreted from pancreatic β cells and maintains functional activity via exosome vesicles [17]. Therefore, it is possible that targeting NCDase in pancreatic β cells will provide a novel therapy for type 2 diabetes mellitus and lipotoxicity.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflicts of interest.


