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Carnitine palmitoyltransferase 1 (CPT1) alleviates oxidative stress and apoptosis of hippocampal neuron in response to beta-Amyloid peptide fragment A β ₂₅₋₃₅

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ABSTRACT

CPT1C, which is expressed in hippocampus, influences ceramide level, endogenous cannabinoid and oxidation process, as well as plays an important role in various brain functions such as learning. This study aimed to investigate the role of CPT1C in Alzheimer's disease (AD) and its underlying mechanism. We established a model of Alzheimer's disease in vitro by exposing primary hippocampal neurons to beta-Amyloid peptide fragment 25–35 (A β ₂₅₋₃₅). The cell viability, lactate dehydrogenase (LDH) level, expressions of reactive oxygen species (ROS), malondialdehyde (MDA) and superoxide dismutase (SOD) were detected using Cell Counting Kit-8 (CCK-8), LDH assay, ROS kits, malondialdehyde (MDA) kits and SOD kits, respectively. Moreover, the expression of oxidative stress-related proteins as well as the expressions of amyloid precursor protein (App), p-Tau and β -site APP-cleaving enzyme1 (Bace-1) were measured using quantitative reverse transcription PCR (RT-qPCR) and western blot. Tunel and western blot were adopted to detect apoptosis as well as its related proteins. After the treatment of peroxisome proliferator-activated receptor alpha (PPAR α), CPT1C expression was detected with the application of RT-qPCR and western blot. CPT1C expression was reduced in A β ₂₅₋₃₅-induced HT22 cells. Overexpression of CPT1C relieved cell viability and toxic injury as well as attenuated oxidative stress, apoptosis and expression levels of AD marker proteins. Moreover, higher doses of PPAR α agonist activate the expression of CPT1C in A β ₂₅₋₃₅-induced HT22 cells. In conclusion, CPT1C alleviates A β ₂₅₋₃₅-induced oxidative stress, apoptosis and deposition of AD marker proteins in hippocampal neurons, suggesting that CPT1C has favorable effects on alleviating AD and participates in PPAR α activation.

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Introduction

Alzheimer's disease (AD) is referred to as progressive cognitive decline resulting in dementia [1]. According to recent statistics, around 35.6 million people have suffered from dementia in the globe, which may be partially due to the high prevalence of AD [2]. What is beyond people's recognition is that neurodegenerative disease is not confined to the elder but is spreading quickly, resulting in increased financial burden and lowering people's life quality [3]. Current therapeutic methods, including treatment with cholinesterase inhibitors, cannot change the rising trend of illnesses [4].

Carnitine palmitoyl transferase (CPT) system, a multiprotein complex featuring catalytic properties is located in a core represented by CPT1 and

CPT2 in the outer and inner membrane of the mitochondria, respectively [5]. Similar to the canonical CPT enzymes in the sequence of primary amino acid, CPT1C is located in the endoplasmic reticulum of neurons and exerts residual catalytic effects in vitro with palmitoyl-CoA [6,7]. As evidenced by previous studies, depletion of CPT1C made more palmitoyl-CoA available, and mice with CPT1C knockdown exhibited metabolic disturbance like impaired gluconeogenesis and muscle glucose uptake [8]. CPT1C, which is expressed in hippocampus, influences ceramide level, endogenous cannabinoid and oxidation process, and plays an important role in various brain functions such as learning [5]. In addition, some studies held that the deficiency of CPT1C on brain can cause motor dysfunction and behavioral defects [9]. CPT1C is considered to possibly involve in AD

due to its role in energy homeostasis. The onset of AD is associated with insulin resistance, which is modulated by palmitate in the hypothalamus and implicated in increased production of reactive oxygen species (ROS) [5,10–12]. However, the role of CPT1C in AD has remained elusive, which constitutes the pivotal focus of our study.

In the present study, we hypothesize that CPT1C plays a role in AD and is involved in the regulation for oxidative stress, apoptosis and AD markers. The study was designed to decipher the role of CPT1C in an in vitro model of AD and figure out how CPT1C was implicated in AD.

Materials and methods

Cell culture and treatment

Mouse hippocampal neuron (HT22) was provided from Salk Institute (La Jolla, CA, USA). The cells were cultured in Dubelcco's modified eagle medium (DMEM; Gibco, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, NY, USA) and 1% penicillin/streptomycin at 37 C in a humidified incubator with 5% CO₂. Aβ₂₅₋₃₅ was diluted to 1 mM with sterilized saline water and then incubated at 37 C for 7 days before use, as previously described [13]. Thereafter, the HT22 cells were treated with 20 mol/L Aβ₂₅₋₃₅ for 48 h. CPT1C overexpression plasmids (Ov-CPT1C, 1 μg/mL) and its empty vector (Ov-NC) were obtained from Shanghai Integrated Biotech Solutions Co., Ltd (Shanghai, China), and transfection was conducted for 24 h by Lipofectamine 3000 (Invitrogen). Gemfibrozil (Abcam, ab142883), a PPARα agonist [14], was used for the following experiments.

RT-qPCR

RNA from HT22 cells was extracted using a TRIzol® kit in accordance with the manufacturer's instructions. The synthesization with complementary DNA (cDNA) was performed using a PrimeScript RT Master Mix kit (Takara Biotechnology, Co., Ltd.). Gene expression was quantified by quantitative real-time PCR (RT-qPCR) on ABI 7500 PCR system (ABI, USA) using SYBR Green PCR Master Mix. All samples

were measured in triplicate and the mean value was calculated. Quantitative measurements were determined using 2^{-ΔΔCT} method [15], and expression of GAPDH was considered as the internal control.

Western blot

Total proteins were isolated from HT22 cells using RIPA buffer (Beyotime, Shanghai, China). A BCA kit was used to quantify the concentration of protein samples. Subsequently, equal amounts of proteins were subjected to 10% SDS-PAGE gel, followed by the transfer to PVDF membranes (EMD Millipore, Billerica, MA, USA). After being blocked with 5% nonfat milk, the membranes were then incubated with primary antibodies (CPT1C, cat.no. DF12150; SOD1, cat.no. AF5198; SOD2, cat.no. AF5144; Bcl2, cat.no. AF6139; Bax, cat.no. AF0120; cleaved PARP, cat. no. BF9106; APAF-1, cat.no. AF0117; App, cat.no. AF6084; p-Tau, cat.no. AF3148. Affinity, USA) (Bace-1, cat.no. ab183612; GAPDH cat.no. ab8245. Abcam). Thereafter, these blots were incubated with a HRP-conjugated antibody (cat.no. #7074, Cell Signaling Technology, Inc.) at 37°C for 1 h. Proteins were visualized with an enhanced chemiluminescence (ECL) and the expression of protein was normalized to GAPDH.

CCK-8 assay

Briefly, cells were inoculated into 96-well plates at a concentration of 3 × 10⁴/well for 12 h. After indicated treatment, CCK-8 reagent (abcam, England) was added to each well to incubate the cells for another 4 h. Then, the optical density (OD) value at 450 nm was determined using a microplate reader (Molecular Devices, San Jose, CA, United States).

Detection of Superoxide Dismutase (SOD) Activity, Malondialdehyde (MDA) Content, and LDH Release Assay

For the detection of cell SOD activity and MDA content, cells after treatment were cultured in 96-well plates, and then sonicated and centrifuged to obtain the supernatant. The SOD activity and MDA content were then measured according to the manufacturer's protocol (Beyotime, Shanghai,

China) and results were displayed as a multiple relative to the control group. The cell supernatant was collected and used for the determination of LDH activities using LDH Cytotoxicity Assay Kit according to manufacturer's protocol (Beyotime, Shanghai, China).

Measurement of ROS

The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) kit was used for the detection of ROS level in HT22 cells according to manufacturer's guidance (Beyotime, Shanghai, China). Briefly, the cells were seeded in 96-well plates and then processed with working solution for 20 min at 37°C. After washing with serum-free medium for three times, the fluorescence density was detected using a fluorescence microplate (biosys, Germany).

TUNEL assay

The cell apoptosis rate was determined using TUNEL assay kits (Invitrogen; Thermo Fisher Scientific). Briefly, the collected cells were fixed with 4% paraformaldehyde for 30 min incubated with 0.3% Triton X-100 in PBS for 10 min according to the manufacturer's protocol. After staining with DAPI for 30 min, the positive-apoptotic cells were counted under a magnification of 200 × .

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6 (Graph Pad Software, La Jolla, CA). The results were expressed as mean ± standard deviation (SD). One-way ANOVA was used for comparison among multiple groups, followed by Tukey's post hoc test between two groups. $P < 0.05$ indicated statistical significance. Each experiment was repeated at least three times.

Results

CPT1C expression was reduced in $A\beta_{25-35}$ -induced HT22 cells

Despite the fact that CPT1C is ubiquitously presented in various transformed cells and cancers [16], the current strategy we used for the following

study is to detect its expression in vitro. Firstly, we induced the cells by 20 μM $A\beta_{25-35}$, while the control group was not treated with $A\beta_{25-35}$. Results from CCK-8 clearly implied that the cell viability of $A\beta_{25-35}$ -induced HT22 cells was decreased and the effects of A_{25-35} on HT22 cells were in a time-dependent manner (Figure 1(a)). As Figure 1(b) shown, the expression of CPT1C was decreased both at a transcript and at a protein level by $A\beta_{25-35}$ over time.

CPT1C overexpression attenuated cell viability and toxic injury in $A\beta_{25-35}$ -induced HT22 cells

To determine the role of CPT1C, we transfected $A\beta_{25-35}$ -induced HT22 cells with CPT1C overexpression plasmids to observe whether CPT1C expression could manipulate the cellular behaviors in hippocampal neurons. As Figure 2(a) indicated, the expression of CPT1C was greatly increased after $A\beta_{25-35}$ induction. Results from CCK-8 assay indicated that the decreased cell viability of HT22 cells induced by $A\beta_{25-35}$ was reverted by CPT1C overexpression (Figure 2(b)). Furthermore, the relative LDH level in HT22 cells was significantly increased after $A\beta_{25-35}$ induction while CPT1C overexpression reversed the promotive effects of $A\beta_{25-35}$ (Figure 2(c)). The above results illustrated that the overexpression of CPT1C attenuated cell viability and toxic injury in $A\beta_{25-35}$ -induced HT22 cells.

CPT1C overexpression attenuated oxidative stress in $A\beta_{25-35}$ -induced HT22 cells

We then observed if oxidative stress and cell apoptosis were changed after CPT1C was overexpressed in A_{25-35} -induced HT22 cells. According to Figure 3(a), the greatly increased levels of ROS and MDA, as well as in $A\beta_{25-35}$ -induced HT22 cells was suppressed by CPT1C overexpression. In addition, CPT1C overexpression downregulated MDA levels in $A\beta_{25-35}$ -induced HT22 cells but enhanced SOD activities. Besides, SOD1 expression levels at transcription and translation levels were increased by CPT1C overexpression when compared with the cotreatment group of $A\beta_{25-35}$ and Ov-NC. These suggested that CPT1C overexpression exhibited inhibitory effects on oxidative stress in $A\beta_{25-35}$ -induced HT22 cells (Figure 3(b,c)).

CPT1C overexpression decreased the apoptosis of $A\beta_{25-35}$ -induced HT22 cells

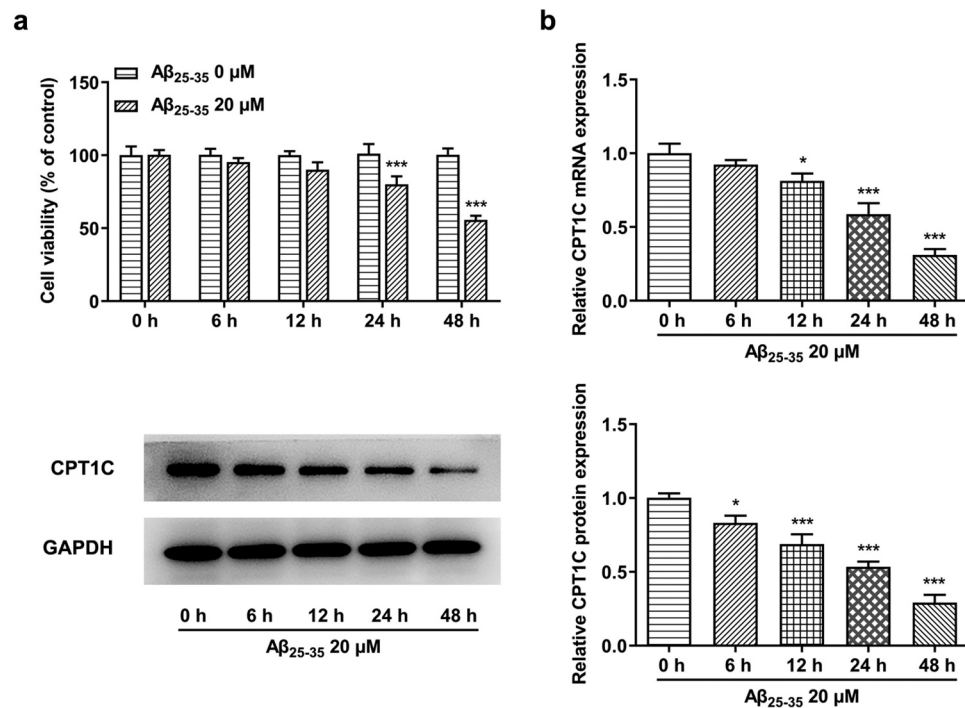


Figure 1. CPT1C expression was reduced in Aβ₂₅₋₃₅-induced HT22 cells. HT22 cells were treated with Aβ₂₅₋₃₅ (20 μM) for 6 h, 12 h, 24 h or 48 h, respectively.

(a) The cell viability of Aβ₂₅₋₃₅-induced HT22 cells was detected using CCK-8 assay. (b) mRNA and protein expressions of CPT1C in Aβ₂₅₋₃₅-induced HT22 cells were measured using RT-qPCR and western blot, respectively. *P < 0.05 and ***P < 0.001 vs. Control group.

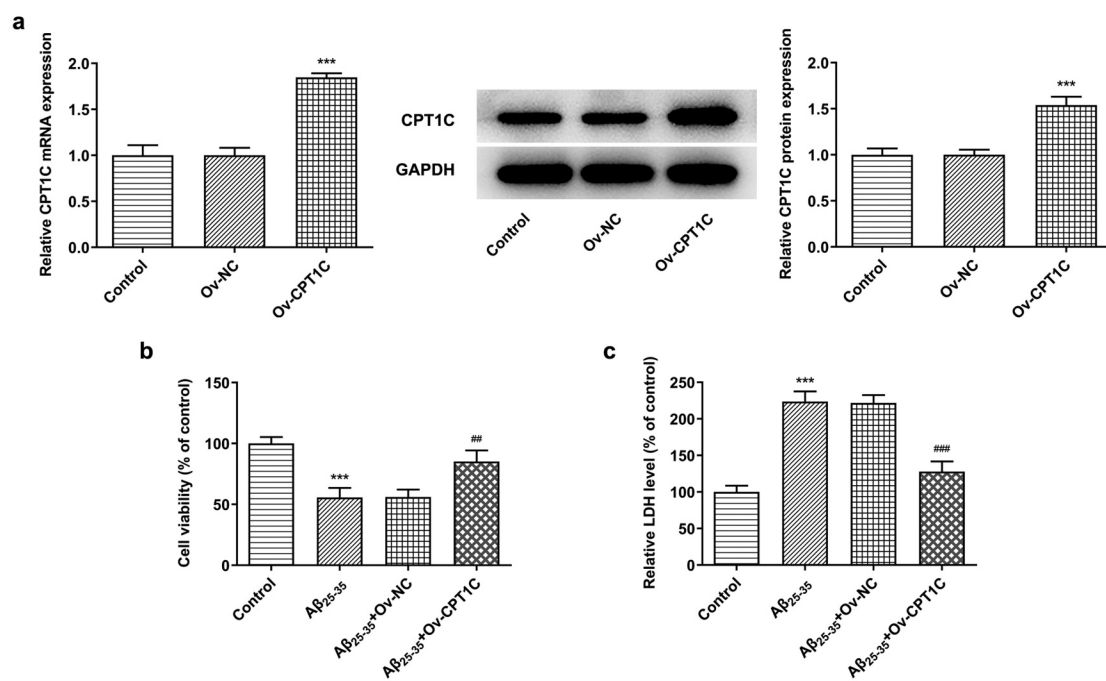


Figure 2. CPT1C overexpression attenuated cell viability and toxic injury in Aβ₂₅₋₃₅-induced HT22 cells. HT22 cells were transfected with Ov-CPT1C or Ov-NC for 24 h, and then treated with Aβ₂₅₋₃₅ for another 24 h.

(a) mRNA and protein expressions of CPT1C were measured using RT-qPCR and western blot, respectively. ***P < 0.001 vs Ov-NC. (b) The cell viability of Aβ₂₅₋₃₅-induced HT22 cells was detected using CCK-8. ***P < 0.001 vs. Control group, ##P < 0.01 vs Aβ₂₅₋₃₅ + Ov-NC. (c) The relative LDH level of Aβ₂₅₋₃₅-induced HT22 cells was checked using LDH assay. ***P < 0.001 vs. Control group, ###P < 0.001 vs Aβ₂₅₋₃₅ + Ov-NC.

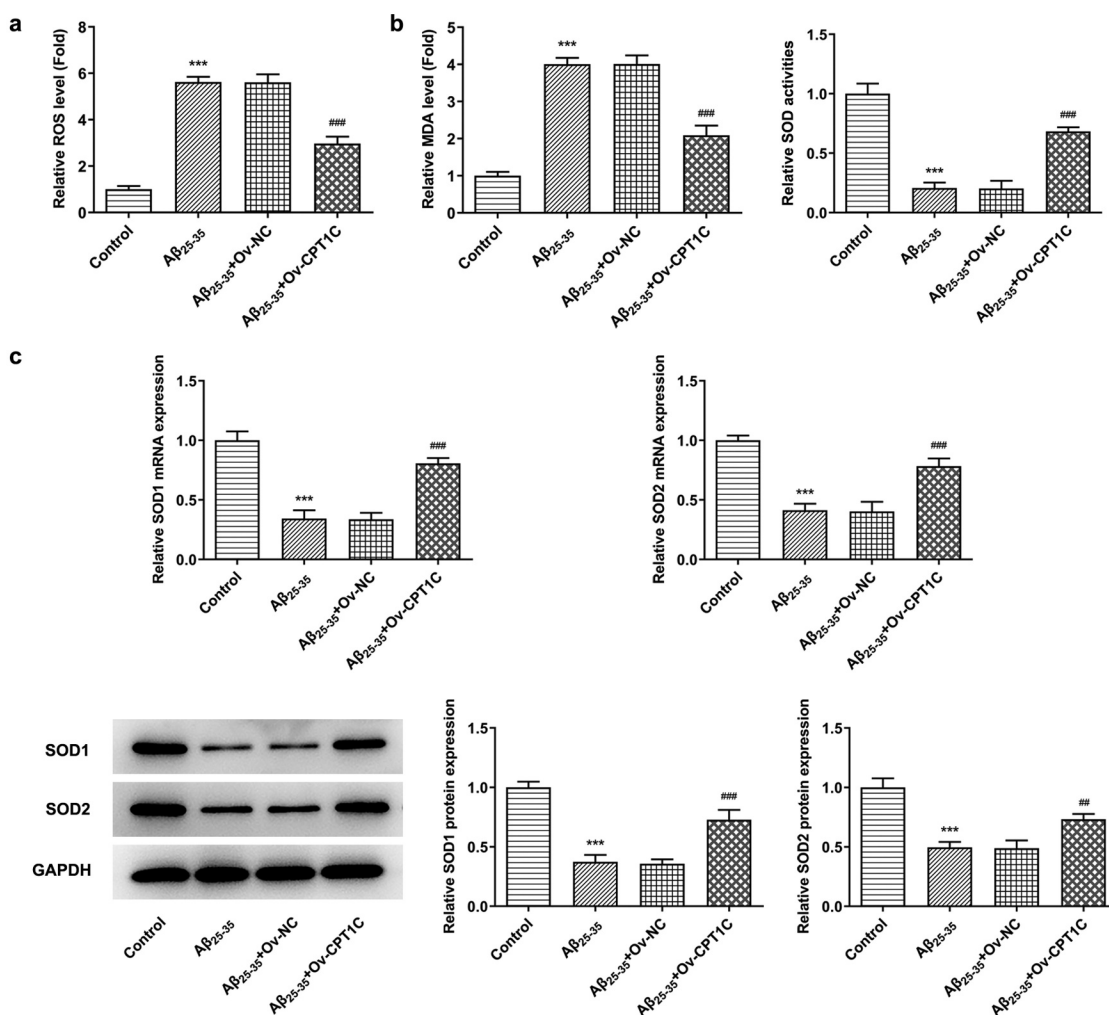


Figure 3. CPT1C overexpression attenuated oxidative stress in A β_{25-35} -induced HT22 cells. Following transfection of Ov-CPT1C or Ov-NC for 24 h, HT22 cells were treated with A β_{25-35} for another 24 h.

(a) ROS expression was detected using ROS kits. (b) MDA levels and SOD activity were measured using MDA and SOD kits, respectively. (c) The relative mRNA and protein expression of SOD1 and SOD2 in A β_{25-35} -induced HT22 cells were measured using RT-qPCR and western blot, respectively. *** $P < 0.001$ vs. Control group, ## $P < 0.01$ and ### $P < 0.001$ vs A β_{25-35} + Ov-NC.

We further determined whether CPT1C regulated apoptosis in HT22 cells treated with A β_{25-35} . As **Figure 4(a)** demonstrated, the apoptosis in HT22 cells was hugely increased by A β_{25-35} induction in comparison with the A β_{25-35} group. However, the increased apoptosis level was then decreased by CPT1C overexpression. Moreover, A β_{25-35} brought about Bcl2 downregulation and Bax, cleaved PARP, and APAF-1 upregulation, which were then reversed by CPT1C overexpression (**Figure 4(b)**). Therefore, we could conclude that overexpression of CPT1C attenuates the apoptosis in hippocampal neurons.

CPT1C overexpression decreased the deposition of AD marker proteins in A β_{25-35} -induced HT22 cells

We next analyzed whether CPT1C overexpression could change the expression of AD markers in A β_{25-35} -induced HT22 cells. With the application of RT-qPCR and western blot, the relative mRNA and protein expressions of App, p-Tau and Bace-1 were measured. Compared with Control, the expressions of App, p-Tau and Bace-1 were greatly upregulated by A β_{25-35} , while CPT1C overexpression reversed the promotive effects of A β_{25-35} on deposition of AD marker proteins, evidenced

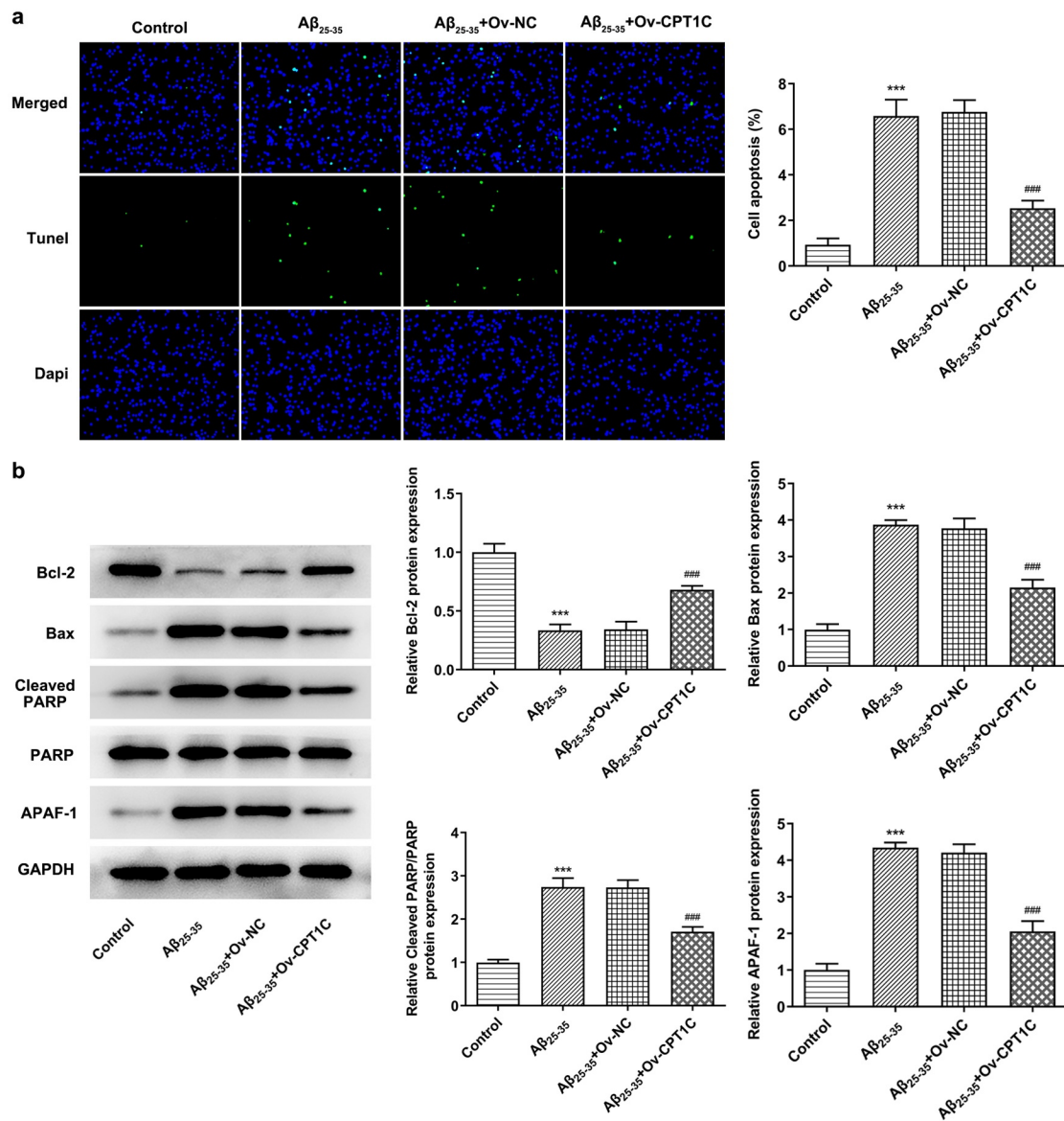


Figure 4. CPT1C overexpression decreased the apoptosis of A β_{25-35} -induced HT22 cells. Following transfection of Ov-CPT1C or Ov-NC for 24 h, HT22 cells were treated with A β_{25-35} for another 24 h.

(a) The apoptosis of A β_{25-35} -induced HT22 cells was evaluated by TUNEL. (b) The expressions of Bcl2, Bax, cleaved PARP and APAF-1 were measured by western blot. *** $P < 0.001$ vs. Control group, ### $P < 0.001$ vs A β_{25-35} + Ov-NC.

by the downregulated expressions of App, p-Tau and Bace-1 in contrast with A β_{25-35} + Ov-NC (Figure 5(a,b)). PPAR α activation could increase CPT1C expression in A β_{25-35} -induced HT22 cells

In order to explore whether CPT1C could be activated by PPAR α , gemfibrozil, an agonist of PPAR α , was used to treat A β_{25-35} -induced HT22 cells. According to Figure 6(a,b), the

expression of CPT1C was significantly diminished by A β_{25-35} in comparison with Control. Studies have noted that PPAR α regulates the malignant behaviors of tumor cells by targeting CPT1C, and PPAR α activation can alleviate the amyloidosis and reverse memory deficits and anxiety in AD [14,17]. After the treatment of gemfibrozil with a dose of 250 μ M, mRNA and protein expressions of CPT1C gained a huge

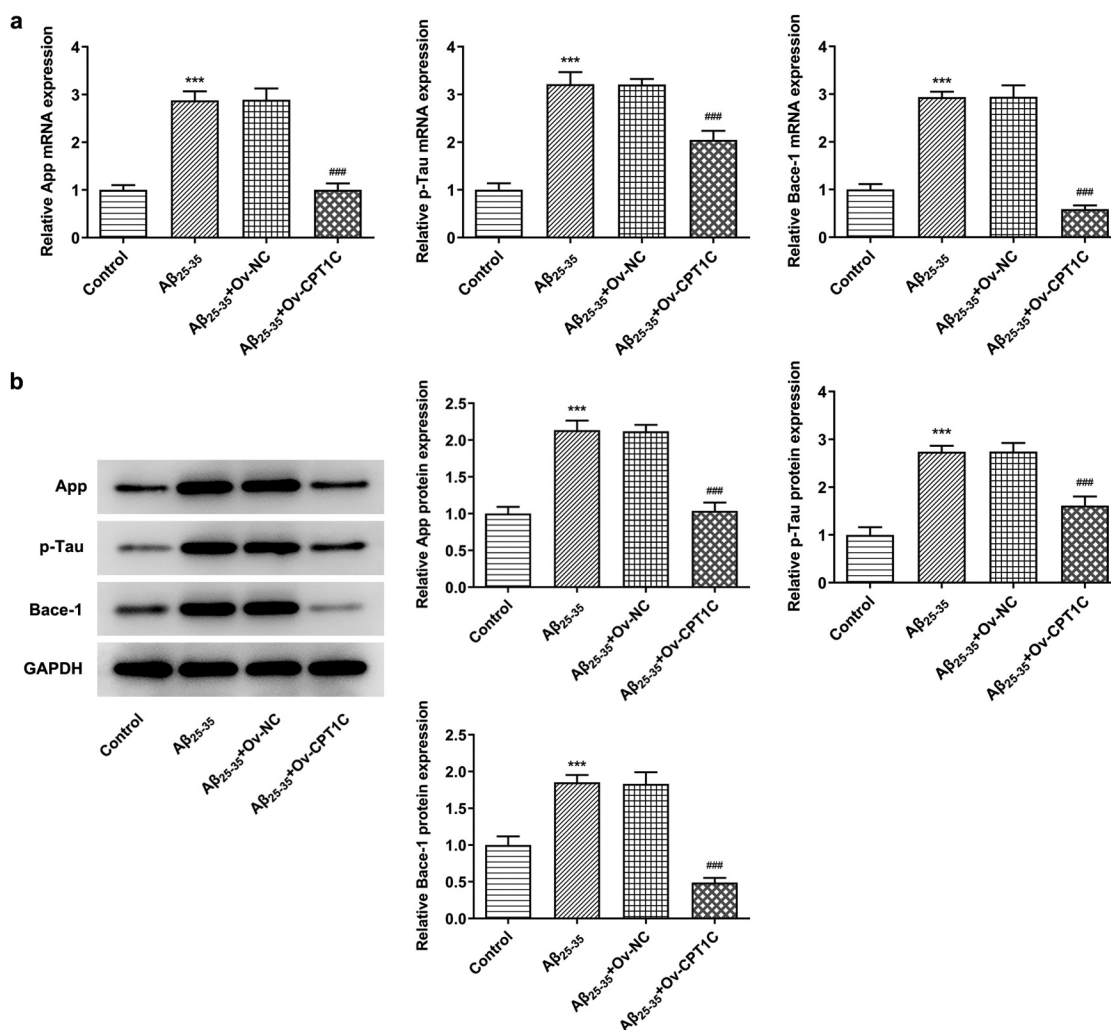


Figure 5. CPT1C overexpression decreased the deposition of AD marker proteins in A β_{25-35} -induced HT22 cells. Following transfection of Ov-CPT1C or Ov-NC for 24 h, HT22 cells were treated with A β_{25-35} for another 24 h.

(a) The mRNA expressions of App, p-Tau and Bace-1 were evaluated using RT-qPCR. (b) The protein expressions of App, p-Tau and Bace-1 were evaluated using western blot. *** $P < 0.001$ vs. Control group, ### $P < 0.001$ vs A β_{25-35} + Ov-NC.

growth, revealing that PPAR α activation could increase CPT1C expression in A β_{25-35} -induced HT22 cells.

Discussion

AD is a neurodegenerative disorder that affects the cognitive functions of human beings, especially the elderly [18]. The aggregation of A β , which has been considered as a major driver of AD progression, was linked to the severity of cognitive deficits [19,20]. Similar to the induction procedures conducted by previous studies, this paper used A β_{25-35} to construct the *in vitro* AD model.

The intricate pathology of AD has necessitated novel targeted treatment methods to fight against its invasion [21]. Oxidative stress is a major driver of AD pathophysiology, and antioxidant agents have been highlighted as optimal choices for the inhibition of AD progression [22]. Patients with AD are often found to suffer from oxidative damage to the neuronal tissues [23]. In this study, CPT1C overexpression led to increased SOD expression, and decreased MDA as well as ROS expression in A β_{25-35} -induced HT22 cells. A previous study showed that the loss of CPT1C triggered increased sensitivity of cells to oxidative stress, as demonstrated by the elevation of

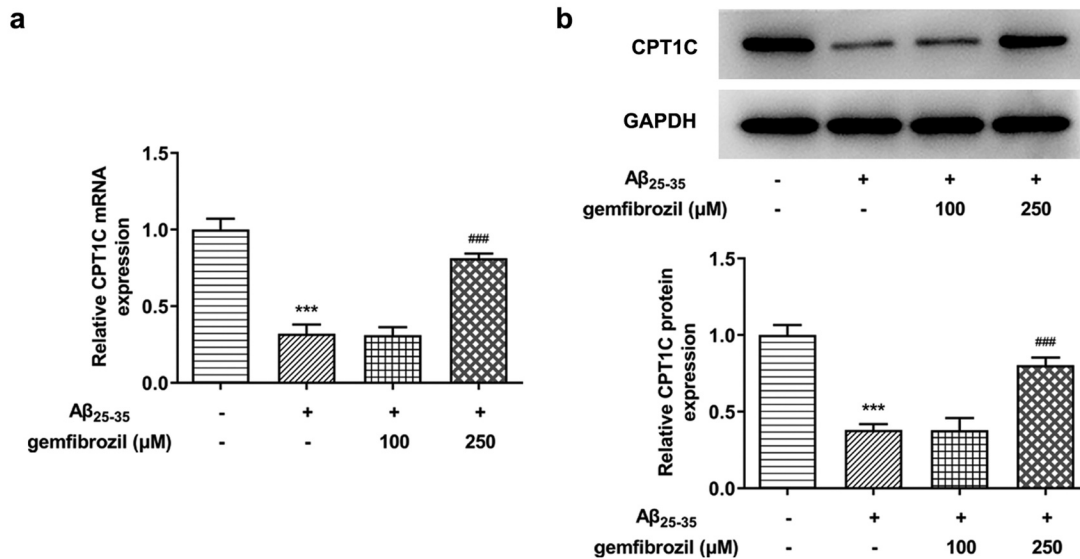


Figure 6. PPAR α activation could increase CPT1C expression in A β_{25-35} -induced HT22 cells. HT22 cells were co-treated with gemfibrozil 100 μ M or 250 μ M, and A β_{25-35} for 48 h.

(a) The relative CPT1C mRNA expression in A β_{25-35} -induced HT22 cells was detected using RT-qPCR. (b) The protein expression of CPT1C expression in A β_{25-35} -induced HT22 cells was detected using western blot. *** $P < 0.001$ vs. Control group, ### $P < 0.001$ vs A β_{25-35} + gemfibrozil 100 μ M.

ceramides, a major regulator of oxidative stress [24]. The findings in this study were consistent with the above-mentioned study. Mounting evidence has noted that apoptosis, which is also called programmed cell death, partakes in the AD-associated nerve cell death [25,26]. Thus, the changes of apoptosis were observed after CPT1C was overexpressed in A β_{25-35} -induced HT22 cells. Importantly, the apoptosis elevated by A β_{25-35} was attenuated by CPT1C overexpression. Moreover, it was found that overexpression of CPT1C abolished the promotive effects of A β_{25-35} on enhancing the expression of AD markers. Thus, decreased CPT1C by A β_{25-35} treatment was able to induce oxidative stress and apoptosis, and CPT1C could be involved in AD pathology.

Peroxisome proliferators activated receptors (PPARs) are a family of ligand-regulated nuclear receptors regulating transcriptions via a complex mechanism [27–29]. Evidence has shown that apart from the modulation of mitochondria metabolism, PPAR α participates in amyloid beta precursor protein (APP) in the brain and it may also influence Tau protein phosphorylation via A [30]. It is well acknowledged that PPAR α played an essential role in neuronal cells [31]. Moreover, PPAR α polymorphism may be

deemed as a risk factor for AD [32]. Thus, we speculated that there might be a close connection between CPT1C and PPAR α in regulating the progression of AD. The administration of gemfibrozil at higher dosage increased the expression of CPT1C inhibited by A β_{25-35} , which tallied with the idea proposed by several experts that alteration of PPAR α signaling may lead to activation of APP metabolism that contributed to AD pathogenesis [30]. Meanwhile, some held that the activation of PPAR α , which was found to be downregulated in AD brain, may also alleviate the inductive effects of A β_{25-35} on AD [30]. Besides, the recognized association of PPAR α and CPT1C in our study was also found by other study which revealed that PPAR α was able to activate the transcription of CPT1C promoter to regulate cell proliferation [17]. Taken together, the inhibition of PPAR α in HT22 cells under A β_{25-35} stimulation is the most cause of decreased CPT1C expression, further inducing oxidative stress and apoptosis.

Conclusion

A β_{25-35} induced decreased CPT1C, thereby leading to oxidative stress and apoptosis. The decreased CPT1C could be due to the inhibition of A β_{25-35}

for PPAR α . CPT1C could play a vital role in AD and may provide insight into AD treatment.

Limitation

The limitation is that current therapeutic strategies can only mildly slow but not halt the progression of AD and this paper has suggested favorable outcomes of CPT1C in alleviating AD and its underlying mechanism related to PPAR activation. It is noteworthy that further in vitro and vivo, and clinical studies regarding the role of CPT1C in AD are still needed for the better management of this disorder.

Highlights

1. HT22 cells expressed lower CPT1C levels when subjected to A β ₂₅₋₃₅ stimulation.
2. CPT1C participates in A β ₂₅₋₃₅-induced oxidative stress and apoptosis, along with reducing the expression levels of AD-related markers.
3. A β ₂₅₋₃₅ could inhibit PPAR α to decrease CPT1C expression, thereby involving in AD pathology.

Disclosure statement

The authors declare that they have no competing interests.

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