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Palmitate induces lipoapoptosis in Schwann cells through ROS generation-mediated STAMP2 downregulation

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ABSTRACT

Free fatty acids (FFAs) are considered the principal inducers of lipotoxicity, leading to cell dysfunction and/or cell death. Lipotoxicity in Schwann cells (SCs) damages neurons, which may be associated with peripheral neuropathies and axon degeneration. However, the molecular mechanism by which FFAs exert lipotoxicity in SCs remains to be established. In the present study, we demonstrate that palmitate exerts lipotoxicity in SCs through apoptosis and that palmitate-induced lipotoxicity in SCs is mediated through reactive oxygen species (ROS) generation. We observed that the six-transmembrane protein of prostate 2 (STAMP2), which plays a pivotal role in lipid homeostasis, is expressed in SCs. We further demonstrate that palmitate induces lipoapoptosis in SCs through ROS generation-mediated STAMP2 downregulation and that STAMP2 depletion accelerates the palmitate-exerted lipoapoptosis in SCs, indicating that STAMP2 confers on SCs the ability to resist palmitate-induced lipotoxicity. In conclusion, palmitate induces lipoapoptosis in SCs through ROS generation-mediated STAMP2 downregulation. Our findings indicate that ROS and STAMP2 may represent suitable targets for pharmacological interventions targeting lipotoxicity-associated peripheral neuropathies and axon degeneration.

1. Introduction

Lipid imbalance is a key metabolic alteration associated with metabolic syndrome and obesity. In hyperlipidaemic states, lipids abnormally accumulate in non-adipose tissues. While adipocytes have the proper biochemical machinery to store and safely utilize high amounts of lipids, non-adipose tissues are vulnerable to lipid overload. Free fatty acids (FFAs), which are elevated in metabolic syndrome and obesity, are considered the principal generators of lipotoxicity, leading to cell dysfunction and/or cell death [1]. Lipotoxicity has been demonstrated in various organs and tissues [2–4]. Nerve cells are also exposed to multiple pathologies that lead to lipid overload and lipotoxicity, which can result in lipid peroxidation and lipotoxicity [5–7].

Schwann cells (SCs) are principal glial cells of the vertebrate peripheral nervous system (PNS). SCs maintain PNS integrity by processes including axon myelination, axonal regeneration, and directional guidance of axons [8]. As SCs are essential for the function and survival of PNS neurons, their dysfunction causes neurodegenerative diseases [9]. Demyelination, a major consequence of damaged SCs, causes axonal degeneration and neuronal dysfunction by altering organization and dynamics of the axonal cytoskeleton [10–12]. A study showed that remodeling of SC lipid metabolism followed by mitochondrial dysfunction results in the depletion of important lipid myelin components, which leads to subsequent demyelination [13]. However, axonal degeneration

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without demyelination can occur by mitochondrial dysfunction in SCs or specific gene mutations [14–16]. Recent studies show that the energy needs of axons largely depend on metabolic support from SCs [17,18]. Therefore, dysfunction of SCs damages neurons both directly by loss of myelination and indirectly by loss of metabolic supports. While lipotoxicity in neurons is established by numerous studies, information on lipotoxicity in SCs is limited [19,20].

In this study, we identified that palmitate induces lipoapoptosis in SCs through reactive oxygen species (ROS) generation and the resulting downregulation of six-transmembrane protein of prostate 2 (STAMP2).

2. Materials and methods

2.1. Schwann cell line culture

RT4-D6P2T cells were grown on monolayers at 37 °C in a 5% CO_2 humidified atmosphere. RT4-D6P2T cells purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) were grown in complete high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA).

2.2. Animals and primary SC culture

All animal study procedures were approved by the Committee on Animal Investigations at Dong-A University (DIACUC-16-15). Sprague-Dawley rats were obtained from Samtako (Osan, Korea). Sciatic nerves from postnatal day 5 rats were dissected, digested enzymatically with collagenase (Sigma, 200 μg/ml) and trypsin (Gibco, 0.05%), and plated onto poly-D-lysine- (PDL, Sigma) coated culture dishes in DMEM with 10% FCS. The cells were washed in HBSS containing 40 mg/l gentamicin (Sigma). The cells were then seeded at a density of 10×10^5 cells/75 cm^2 tissue culture flask for protein and mRNA expression studies.

2.3. Treatment with FFAs and other reagents

Twenty-four hours after RT4-D6P2T cells were sub-cultured the original medium was removed. The cells were washed with PBS and then incubated in the fresh medium with 2% FBS for 24 or 48 h. Palmitate and oleate (Sigma) were dissolved in absolute ethanol at a concentration of 500 mM and diluted to their final concentrations with the appropriate concentration of 1% (w/v) of FFA-free BSA (Sigma). Controls were incubated with equal concentrations of FFA-free BSA containing ethanol. The cells were pre-treated with caspase inhibitor I (zVAD-fmk, Calbiochem, San Diego, CA, USA) and N-acetyl-L-cysteine (NAC, Sigma) for 2 h and were further exposed to palmitate for 48 h.

2.4. Cell viability assay

An automated trypan blue exclusion assay was performed. Total cells and trypan blue-stained (i.e., nonviable) cells were counted, and the percentage of viable cells was calculated using the Vi-Cell cell counter (Beckman Coulter, Miami, FL, USA).

2.5. Nuclear morphology assay for apoptosis

Cells were harvested and washed with PBS. They were fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were washed with PBS twice and stained in 4 μg/ml Hoechst 33342 (Sigma) for 30 min at 37 °C. Stained cells were coated onto clean, lipid-free glass slides and mounted with a cover glass. The samples were observed and photographed under an epifluorescence microscope (Axiophot, Zeiss, Germany). The number of cells that showed condensed or fragmented nuclei was determined by a blinded observer from a random sampling of 250–300 cells per experiment.

2.6. Western blot analysis

Cells (2×10^6) were washed twice with ice-cold PBS. Cells were resuspended in lysis buffer [200 μl of ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris–Cl (pH 7.6), 0.5% Triton X-100, protease inhibitor cocktail)] and incubated at 4 °C for 30 min. The lysates were centrifuged at 14,000 rpm for 20 min at 4 °C. The concentrations of protein in the cell lysates were measured with the Bradford protein assay reagent (Bio-Rad, Hercules, California, USA). Then, 20 μg of protein was loaded onto 8–15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. The separated proteins were transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) and probed with the following primary antibodies: anti-cIAP1, anti-cIAP2, anti-Survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase 3, anti-caspase 7, anti-XIAP (Cell Signaling Technology, Beverly, MA, USA), anti-β-actin (Sigma), and anti-STAMP2 (Proteintech, Rosemont, IL, USA). Bound primary antibodies were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling). Immunostaining with antibodies was carried out using the Super Signal West Pico (Thermo Fisher Scientific, Waltham, MA, USA) enhanced chemiluminescence substrate, and detected with LAS-3000PLUS (Fujifilm Life Science, USA).

2.7. Flow cytometric measurement of apoptosis, mitochondrial membrane potential (MMP), mitochondrial mass and mitochondrial ROS

For quantitation of apoptosis, cells were incubated with 1 ml of annexin V-binding buffer containing 5 μl of annexin V-FITC (Sigma) conjugate and 10 μl of propidium iodide solution. After incubating for 10 min at room temperature, the cells were analyzed by flow cytometry. For measurement of the MMP, cells were incubated with 1–5 μg/ml 3,3′-dihexyloxacarbocyanine iodide (DiOC6, Thermo) for 15 min at 37 °C. For quantitation of mitochondrial mass contents, the cells were incubated in 200 nM MitoTracker Green (Thermo) for 30 min at 37 °C. For quantitation of mitochondrial ROS, the cells were incubated in 5 μM MitoSOX for 30 min at 37 °C. Data were acquired and analyzed with an Epics XL (Beckman Coulter, Miami, FL, USA).

2.8. Staining of lipid droplets (LDs) and mitochondria, confocal microscopy and quantification

Cells cultured on coverslips were incubated with 1 μg/ml BODIPY 493/503 (Molecular probe, Eugene, OR, USA) for 10 min or 300 nM MitoTracker Red (Thermo Fisher Scientific) for 30 min, and counterstained with Hoechst 33342 for 30 min. Fluorescent images were observed and analyzed using a Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany). Twenty cells from each experiment or animal were observed. Quantification of BODIPY 493/503 fluorescent intensity in the confocal images was performed using ZEN Blue analysis software.

2.9. siRNA transfection and combination palmitate treatment

Rat STAMP2 siRNA (SMARTpool; L-105419-02-0050) was

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