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cPLA2 and desaturases underlie the tau hyperphosphorylation offset induced by BACE knock-down in neuronal primary cultures

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ABSTRACT

Inflammation has been suggested to play early and perhaps causative roles in Alzheimer's disease (AD) pathogenesis possibly in part by the overactivation of the aspartic acid protease named β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1), which is responsible for the β-amyloid cascade. We have described that BACE1 is involved in the lysophosphatidylethanolamine (LPE) (18:1/20:4/22:6) upregulation associated with tauopathy and inflammation signaling (cPLA2/arachidonic acid/COX2) in a triple transgenic model of Alzheimer's disease, where BACE1 silencing reversed the imbalanced profile and produced cognitive function improvement. In this study, we analyze the role of cPLA2 and desaturases (SCD1, FAD6) in the BACE1 knock-down-induced protective action under a glutamate excitotoxicity model. Glutamate (125 μM) produced hyperphosphorylation of tau in cortical primary cultures along with increased apoptotic nuclei, LDH release, and cPLA2 expression, which were all reversed by BACE1-KD. This beneficial effect was reinforced by the silencing of cPLA2 but attenuated by the reduction in SCD1 and partially attenuated by the reduction in FAD6. Inversely, overexpression SCD1 and FAD6 recovered the neuroprotective effect produced by BACE1-KD, which was not achieved by the overexpression of each desaturase alone. These findings suggest that the hyperphosphorylation of tau and the creation of a pro-inflammatory cell environment are blocked in a desaturase-dependent manner by targeting BACE1.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by senile plaques composed of amyloid-β (Aβ) peptides, intracellular paired helical filaments (PHFs) caused by abnormally phosphorylated tau protein, neuroinflammation and neuronal loss. Aβ is generated by the enzymatic processing of the amyloid precursor protein (APP). This process involves the cleavage of APP by β-site APP cleavage enzyme 1 (BACE1) and the γ-secretase complex, which contains presenilin 1 or 2 (PS1 or PS2) as the catalytic subunit to generate the Aβ40 and Aβ42 peptides [1,2]. The amyloid cascade hypothesis suggests that synaptic toxicity and neurotoxicity mediate the creation of the multimeric amyloid-β peptide species and the consequent hyperphosphorylation of tau, PHF formation and cognitive impairment; however, the molecular etiopathogeneses leading to those events are not fully understood.

Recent works suggest that systemic inflammation could exacerbate or even trigger neuronal dysfunction associated with dementia, including impaired insulin signaling, synapse degradation and memory disturbances associated with the release of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (INOS) [1,3,4]. Elevated APP and COX2 production has been linked to calcium-dependent phospholipase A2 (cPLA2) upregulation *in vivo* and *in vitro* in cortical neurons exposed to Aβ [5]. cPLA2 upregulation is closely associated with inflammation [6], excitotoxicity in spinal cord neurons [7] and mood disorders [8]. Additionally, pro-inflammatory arachidonic fatty acid released by cPLA2 is metabolized by COX2 [9]. In addition, elevated cPLA2 activity has been associated with increased tau hyperphosphorylation during inflammation [10], and MAPK regulates cPLA2 activity under lipid peroxidation conditions [11].

Alternatively, our previous studies have suggested that BACE1 may have a more direct action on tauopathy because it decreases the rate of tau phosphorylation, blocking the MAPK pathway in a triple transgenic AD mice model [12]. Additionally, the reduction in the phosphorylation of tau by shBACE1miR was blocked by an inhibitor of autophagosome lipidation of phosphatidylethanolamine (PE), 3-methyladenine (3-MA) [12]. However, our recent research showed that BACE1 silencing prevents cognitive impairment and inflammation; reduces arachidonic

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acid, cPLA2 and COX2; and recovers the mono- and polyunsaturations of the fatty acid composition of PE plasmalogens, such as lyso (LPE) and ether (ePE), in the hippocampus of 3xTgAD mice [13].

Therefore, considering that cPLA2 is the main enzyme involved in the production of plasmalogens [14] and that stearoyl-coenzyme A desaturase 1 (SCD) and fatty acid desaturase 6 (FADS6) are responsible for fatty acid unsaturation; our next question was to analyze the role of cPLA2 and desaturases (SCD1, FAD6) in BACE1-KD-induced protective action and tau hyperphosphorylation reduction in a model of glutamate excitotoxicity.

2. Materials and methods

2.1. Primary neuronal cultures

Cortical samples from Wistar rat embryos (E18–19) were dissected, dissociated and cultured on poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) precoated multiwell plates in Neurobasal medium (Gibco, Rockville, MD, USA), which contained B-27 supplement (Sigma-Aldrich), albumin from a chicken egg (Sigma-Aldrich), N2 human supplement (GIBCO), and a penicillin-streptomycin antibiotic mixture (GIBCO); the samples were cultured at 37 °C in a 5% CO_2 humidified atmosphere for a maximum of 19 days *in vitro* (DIV19). Isolated primary neurons were plated at a low density (52 cell/mm^2) for immunofluorescence and at a high density (1500 cell/mm^2) for Western blotting to assay cPLA2, SCD1 and FADS6.

2.2. ShRNA-miR AAV viral vector, shRNA lentiviral particles, and transduction

The SCR (scrambled) or BACE1 shRNA-miRs (short hairpin RNA in a microRNA backbone) were designed and validated in our previous study [12]. The adeno-associated virus (AAV) particles were obtained from the Davidson Laboratory (University of Iowa Viral Vector Core). The AAV aliquots were dialyzed, and the cells were transduced with 2 μL AAV 2/5 (10 genomes per mL) at a titer of 10^{12} vg/mL for 3 h at 37 °C; the media were subsequently completed with B27 supplemented Neurobasal medium. The transductions were performed at DIV7 and maintained for 12 days posttransduction prior to the administration of inhibitor and the glutamate treatments. cPLA, SCD1 and FADS6 shRNA mouse (m) viral particles containing target-specific constructs that encode 19–25-nt (plus hairpin) shRNAs were used to knock down gene expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Commercial control shRNA viral particles encoding a scrambled shRNA sequence that does not specifically degrade any known cellular mRNA were also used. At DIV12, the cultures were transduced for 7 days with lentiviral particles at a titer of 106 infectious units of virus in Dulbecco's modified Eagle's medium (DMEM) that contained 25 mM HEPES, pH 7.3.

2.3. Transfection and cotransfection of neuron cultures

Neurons were cotransfected with cPLA2, SCD1 and FADS6 CRISPR activation plasmid (m) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with an empty vector in DMEM with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 4 h, the transfection medium was replaced with DMEM containing 10% fetal bovine serum (FBS). Hippocampal neurons were transduced with SCR or BACE1 shRNAmiR at DIV7. Clustered regularly interspaced short palindromic repeats (CRISPR) activation plasmid (m) and empty plasmid were transfected and cotransfected into cortical neurons with Lipofectamine 2000 (Invitrogen) in Neurobasal medium at DIV17. An equimolar mixture of 0.4 μg of DNA with Lipofectamine 2000 was used. At 4 h, the post-transfection medium was changed for the supplemented Neurobasal medium, and the DIV18 neurons were treated with 125 μM glutamate for 20 min (Sigma-Aldrich) to induce excitotoxicity.

2.4. Treatments

DIV18 neurons were treated with 125 μM glutamate for 20 min to induce excitotoxicity [16]. After 20 min, the glutamate was washed out to evaluate the effects. At 24 h postglutamate, the media were collected for use in the LDH assay, and either the protein was collected or the cells were fixed.

2.5. Immunofluorescence

Immunofluorescence analysis was performed as previously described (Posada-Duque et al. [16]). Briefly, the cells were incubated overnight at 4 °C with the following primary antibodies: anti-mouse cPLA2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-goat SCD1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-goat FADS6 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-mouse phospho-tau (Ser202, Thr205) (AT8) (1:1000, Thermo Fisher, CA, USA) antibodies. The Alexa-594 fluorescent antibody was used as a secondary antibody (1:1000, Molecular Probes, Eugene, OR, USA). The nuclei were stained with Hoechst (1:5000, Invitrogen), and the cells were incubated with each primary antibody conjugated with Alexa 594 (1: 2000, Molecular Probes) for 1 h. The cells were washed 4 times in buffer, cover slipped using Gel Mount (Biomeda, Hatfield, PA, USA), and observed under an Olympus IX 81 epifluorescence microscope (Olympus Latin America, Inc., Miami, FL, USA) or a Disk Scan Unit (DSU) Spinning Disc Confocal microscope. No staining was observed when the primary antibodies were omitted. XY images were collected using an Olympus IX 81 microscope with 10× (NA, 0.4), 40× (NA, 1.3) or 60× (NA, 1.42) oil immersion objectives.

2.6. Quantitative image analysis

The percentage of condensed nuclei was obtained using the following formula: [condensed nuclei/(condensed nuclei + normal nuclei)] × 100 (Posada-Duque et al. [16]). The mean area, diameter and intensity of each nucleus were quantified using the automatic measurement/spatial trace feature tool. Condensed nuclei were defined as those possessing an average area equal to or $< 40\,\mu m^2$, a diameter equal to or $< 6\,\mu m$ and a fluorescence intensity equal to or > 120 (AU). Images were analyzed individually and calibrated to a micrometer scale using Image Scope Pro Plus software (Media Cybernetics Inc., Rockville, MD, USA). Analyses were performed for 15 neurons per treatment in each duplicate assay and for at least four independent experiments (n = 3–5).

2.7. LDH release

A cytotoxicity detection kit (Roche) was used to assess the presence of cytotoxicity by measuring the activity of lactate dehydrogenase (LDH) released by cultures. The culture medium was recovered one day after glutamate treatment (DIV 19); LDH activity was determined by measuring NADH absorption as the linear rate of consumption during pyruvate reduction to lactate using a spectrophotometer. The cytotoxicity was calculated for a given test condition using the following equation: cytotoxicity (%) = [(A − low control)/(high control − low control)] ∗ 100, where A is the mean LDH activity measured in medium samples from three wells per duplicate that were subjected to the test condition, low control is the LDH activity released from untreated normal cells, and high control is the maximum LDH activity released in all cells (cells treated with 1% Triton X-100 for 24 h).

2.8. Western blotting

After treatment, neuronal cultures were homogenized in lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.4), 10% glycerol, 1 mM EDTA, 1% NP40, and 1 mg/mL inhibiting cocktail proteases). Sodium dodecyl