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Research Paper

Inhibition of sphingomyelin synthase 1 ameliorates alzheimer-like pathology in APP/PS1 transgenic mice through promoting lysosomal degradation of BACE1

Mei-Hong Lu[a,b], Wen-Li Ji[a,b], De-En Xu[c], Pei-Pei Yao[a,b], Xiu-Yun Zhao[a,b], Zhao-Tao Wang[d], Li-Pao Fang[a,b], Rui Huang[a,b], Li-Jun Lan[a,b], Ji-Bo Chen[a,b], Ting-Hua Wang[e], Li-Hua Cheng[d], Ru-Xiang Xu[d], Chun-Feng Liu[a,b], Luigi Puglielli[f], Quan-Hong Ma[a,b,d,*]

[a] Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho- Diseases, Institute of Neuroscience, Soochow University, Suzhou 215004, China
[b] Department of Neurology and Suzhou Clinical Research Center of Neurological Disease, the Second Affiliated Hospital of Soochow University, Suzhou 215004, China
[c] Department of Neurology, The Second People's Hospital of Wuxi, Wuxi 214002, China
[d] Affiliated Bayi Brain Hospital, Military General Hospital of Beijing PLA, Southern Medical University, Beijing 100700, China
[e] Institute of Neuroscience, Kunming Medical University, Kunming 650500, China
[f] Department of Medicine and Wisconsin Alzheimer's Disease Research Center, University of Wisconsin-Madison, Madison, WI 53705, USA

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ABSTRACT

Sphingolipids emerge as essential modulators in the etiology of Alzheimer's disease (AD) with unclear mechanisms. Elevated levels of SM synthase 1 (SMS1), which catalyzes the synthesis of SM from ceramide and phosphatidylcholine, have been observed in the brains of Alzheimer's disease (AD), where expression of β-site APP cleaving enzyme 1 (BACE1), a rate limiting enzyme in amyloid-β (Aβ) generation, are upregulated. In the present study, we show knockdown of SMS1 via andeno associated virus (serotype 8, AAV8) in the hippocampus of APP/PS1 transgenic mice, attenuates the densities of Aβ plaques, neuroinflammation, synaptic loss and thus rescuing cognitive deficits of these transgenic mice. We further describe that knockdown or inhibition of SMS1 decreases BACE1 stability, which is accompanied with decreased BACE1 levels in the Golgi, whereas enhanced BACE1 levels in the early endosomes and the lysosomes. The reduction of BACE1 levels induced by knockdown or inhibition of SMS1 is prevented by inhibition of lysosomes. Therefore, knockdown or inhibition of SMS1 promotes lysosomal degradation of BACE1 via modulating the intracellular trafficking of BACE1. Knockdown of SMS1 attenuates AD-like pathology through promoting lysosomal degradation of BACE1.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by progressive memory decline as well as aberrant behavior. Amyloid-β (Aβ), especially its oligomeric form, is the initiator to trigger the pathological processes in AD such as neuroinflammation, loss of synapses and cognitive deficits (Musiek and Holtzman, 2015; Yang et al., 2017). β-secretase (β-site APP cleaving enzyme 1, BACE1) is the rate limiting enzyme in Aβ generation, which cleaves APP together with γ-secretase to generate Aβ. BACE1-deficient neurons and AD transgenic mice produce no soluble Aβ and Aβ plaques (Cai et al., 2001; Luo et al., 2001). Mutation of APP at A673T which suppresses cleavage by BACE1 exhibits cognitive benefit in human (Jonsson et al., 2012). Therefore, inhibiting BACE1 is one of prime targets of AD therapy. It is worth noting that elevated levels and activity of BACE1 have been detected in the brains of AD patients with unclear mechanisms (Holsinger et al., 2002; Yang et al., 2003). Thus, identification of molecular pathways regulating BACE1 expression or activity may bring novel targets or strategies for AD therapy.

"Lipid granule accumulation" was originally described by Alois Alzheimer as the third hallmark of AD brain (Foley, 2010). Recent studies further reveal a close link of lipids to AD pathogenesis. A number of genes involved in lipid metabolism are genetically associated with late onset AD (Naj et al., 2011). Among them, E4 isoform of apolipoprotein E, which encodes a lipid transport protein, is the strongest genetic risk factor for AD (Bertram and Tanzi, 2008). Cholesterol, which exhibits abnormal levels in the brains of both of AD patients and transgenic mice (Walter and van Echten-Deckert, 2013),

* Corresponding author at: Block 402, Ren-Ai Road 199, Suzhou, Jiangsu 215021, China.
E-mail address: maquanhong@suda.edu.cn (Q.-H. Ma).

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regulates Aβ production through modulating γ-secretase activity (Di Paolo and Kim, 2011; Walter and van Echten-Deckert, 2013). Sphingolipids, which modulates biological processes including cell growth, differentiation and senescence, shows altered levels in the brains of AD patients (van Echten-Deckert and Walter, 2012). Sphingomyelin (SM) and ceramide are two major species of sphingolipids, which can convert to each other by SM synthase (SMSs) and sphingomyelinase (SMase) respectively. The levels of ceramide and SMase increase in the brains of both AD model mice and patients (Jana and Pahan, 2004; Grimm et al., 2011; Dinkins et al., 2016). Ceramide promotes stability of BACE1 (Costantini et al., 2007), the generation and aggregation of Aβ (Geekiyanage and Chan, 2011; Dinkins et al., 2016), thus being taken as a risk factor of AD. Inhibition of neutral SMase2 ameliorates AD pathology and improves cognition in AD transgenic mice through reducing exosome secretion, which is enriched with ceramide (Dinkins et al., 2016).

Sphingomyelin synthases (SMSs), which mainly include SMS1 and SMS2, are the last enzymes in the sphingomyelin (SM) biosynthetic pathway. SMSs use ceramide and phosphatidylcholine (PC) as substrates to produce SM and diacylglycerol (DAG). SMS1 is mainly located in the Golgi apparatus, while most SMS2 resides at the plasma membrane (Tafesse et al., 2006). SMS1 shows a capability to produce higher SM content than SMS2 (Shakor et al., 2011). Overexpression of SMS1/2 increases the levels of SM, DAG and ceramide (Ding et al., 2008). It is worth noting that increased levels of SMS1, but not of SMS2, in the hippocampus of AD patients. Inhibition of SMS using a SMS inhibitor, D609, reduces Aβ levels in culture medium with an unknown mechanism (Hsiao et al., 2013). Moreover, elevated SM levels have been observed in the plasma, cerebrospinal fluid (CSF) and brains of AD patients and showed correlation with amyloid plaques (Pettegrew et al., 2001; Chan et al., 2012; Kosicek et al., 2012; Hsiao et al., 2013). In contrast, some studies have observed decreased SM levels in AD brains (Jana and Pahan, 2004; Yang et al., 2004; Grimm et al., 2005; He et al., 2010). Despite of these conflicting observations, in comparison to ceramide, the function of other sphingolipids such as SM in the etiology of AD remains unknown. Therefore, it is worth investigating that the functions of SMS1 in AD pathogenesis, which may help us understand the roles of SMS1-modulated sphingolipids in AD etiology.

We herein show that knockdown of SMS1 by injecting of AAV8 encoding SMS1 shRNA into the hippocampus of APP/PS1 mice, which overexpress mutant human APP and PS1 (Deng et al., 2016), attenuates AD-like pathology such as accumulation of Aβ, neuroinflammation, loss of synapses and cognitive deficits of these mice. We further observe inhibition or knockdown of SMS1 enhances lysosomal degradation of BACE1. We describe inhibition or knockdown of SMS1 causes BACE1 to distribute less in the Golgi, whereas to accumulate in the early endosomes and lysosomes. Thus, we conclude that inhibition of SMS1 promotes lysosomal degradation of BACE1 through regulating the intracellular trafficking of BACE1.

2. Experimental procedures

2.1. Mice

Mice were housed at the SPF Animal Center of Soochow University at 20 °C with a 12-h light/12-h dark cycle (lights on at 6:00 am and off at 6:00 pm).They were maintained in cages with free access to food and water. Male APP/PS1 transgenic mice that co-express mutant human *APP* and *PS1* (Jackson Laboratory, 004462) were used for behavioral tests. All experiment procedures followed the guideline approved by the Institutional Animal Care and Use Committee of Soochow University. After behavior test, the mice were sacrificed by dislocation, the brains were taken for subsequent sectioning or protein extraction.

2.2. Antibodies

Anti-GAPDH (CMCTAG, USA), anti-Aβ (6E10, Convance, USA), anti-Iba1 (Wako, Japan), anti-BACE1 (CST, USA), anti-SMS1 (Proteintech, USA), anti-lysenin (Peptide Institute, Japan), rat anti-neprilysin (R&D system, USA), Alexa Fluor 555, 488 and 694-conjugated antibodies (Invitrogen, USA). Anti-γ-tubulin, anti-HA, anti-APP and horseradish peroxidase (HRP)-conjugated antibodies were purchased from Sigma (USA). Anti-GFAP, anti-synaptophysin, anti-LAMP1, anti-EEA1, anti-GM130 and anti-IDE were purchased from Abcam (UK).

2.3. Cells and siRNA

HEK293 cells stably expressing BACE1-HA were cultured in medium containing 40 μg/ml hygromycin B. Human SMS1 and SMS2 siRNAs are as following: SMS1 sense: 5'-CUACACUCCCAGUACCUGG-3′; SMS2 sense: 5'-ACCUGUUGCACCGAUAUUCAA-3′.

For BACE1 degradation analysis, HEK293 cells were cotransfected with BACE1-HA and SMS1 siRNA for 12 h, followed by treatment with 40 μg/ml cycloheximide (CHX). The cells were harvested at different time points after treatment.

2.4. AAV vector administration

AAV8, which encodes EGFP under CMV promoter and either scrambled shRNA (NC) or a SMS1 shRNA sequence under U6 promoter were purchased from Heyuan Biotech. Ltd. Co. (Shanghai, China). One microliter of AAV8 (1×10^{13} vg/ml) were injected bilaterally into the dorsal hippocampus (−2.0 mm AP, ± 1.7 mm ML, −2.0 mm DV from bregma) of 4 month-old mice using a stereotaxic apparatus (RWD Life Science, China) via a microprocessor controlled minipump (Longer Pump, UK) at a rate of 0.2 μl/min. Behavioral tests were performed 3 months after AAV8 injection.

2.5. Immunofluorescence staining and quantification

Immunofluorescence staining was performed as described (Zhang et al., 2014; Deng et al., 2016). The quantification of immunofluorescence was performed as described (Zhang et al., 2014). The images were converted into 8-bit images and binarized after subtracting the background noise using NIH Image J software. Mean fluorescence intensities (MFIs) of synaptophysin, GFAP, Iba1 and lysenin was measured and calculated by dividing the MFI units by the area of outlined regions. The intensity threshold was set and kept constant for all images analyzed. The numbers of Aβ plaques per section were counted. The size of Aβ plaques was quantified and expressed as the areas of Aβ plaques being divided by the total areas of the cortex or hippocampus. For quantification, the sections from five mice per group and at least eight hippocampal sections per mouse were analyzed ($n = 5$).

The colocalization between BACE1 and LAMP1/GM130/EEA1 in cultured cells and hippocampal sections was analyzed by Image J by quantifying the Pearson's correlation coefficient. Total 120 cells per group from 4 replicated experiments ($n = 4$) were analyzed. 60–80 amyloid plaques per group of mice (four mice per group, n = 4) were analyzed.

2.6. qRT-PCR analysis

Real time PCRs were carried out with SYBR Green 1 (Roche, Switzerland) using the following primers pairs: human *BACE1* (5′-ACCAACCTTCGTTTGCCCAA-3′; 5′-TCTCCTAGCCAGAAACCATCAG-3′); mouse *BACE1* (5′-GGAACCCATCTCGGCATCC-3′; 5′-TCCGATTCCTCGTCGGTCTC-3′); human *SMS1* (5′-CAGCATCAAGATTAAACCCAACG-3′; 5′-TGGTGAGAACGAAACAGGAAAG-3′); mouse *SMS1* (5′-GAAGGAAGTG GTTTACTGGTCAC-3′; 5′-GACTCGGTACAGTGGGGGT-3′); human *SMS2* (5′-TCCTACGAACACTTATGCAAGAC-3′;