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Research article

Anti-neuroinflammatory effects of SLOH in Aβ-induced BV-2 microglial cells and 3xTg-AD mice involve the inhibition of GSK-3β

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ABSTRACT

Neuroinflammation has been observed in post-mortem Alzheimer's disease (AD) brains which could be due to Aβ interacting with microglia and astrocytes. SLOH, a carbazole-based fluorophore, was shown to bind to Aβ peptides. Herein, we investigated the anti-neuroinflammatory effects of SLOH using a BV-2 microglial cell model and a triple transgenic AD (3xTg-AD) mouse model. BV-2 cells were incubated with Aβ in the presence of SLOH for 24 h. The levels of pro-inflammatory and anti-inflammatory cytokines were determined. Moreover, 3xTg-AD mice were administrated with SLOH (2 mg kg^{-1}) for one month. The mice were then sacrificed and the brains were used to assess the levels of pro-inflammatory, anti-inflammatory cytokines and the activation of ionized calcium-binding adapter molecule 1 (Iba1). BV-2 cell studies suggested that SLOH reduced the production and mRNA levels of pro-inflammatory cytokines TNF-α, IL-1β, COX-2, iNOS, and increased IL-10. Animal study confirmed that SLOH reduced the production of pro-inflammatory cytokines and increased the level of anti-inflammatory cytokine. Moreover, SLOH inhibited the activity of GSK-3β. In 3xTg-AD mouse model, SLOH treatment significantly decreased the number of Iba1-positive cells in mouse brains. Our results demonstrated that SLOH significantly attenuated the neuroinflammation through down-regulating the activity of GSK-3β.

1. Introduction

Alzheimer's disease is a serious chronic disease affecting many elderly people [1]. It is characterized by cognitive deficits. As the disease progresses to advanced stages, some AD patients suffer from deprivation of body functions including irritability, aggression, problems with speaking and writing. Due to the lacking of effective treatment, AD has brought severe epidemiologic and economic impacts. In addition to intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques, neuroinflammation is a prominent feature in the modulation of AD processing. The nervous system is formed by two major cell types, namely neurons and glial cells. Neuroinflammation is mainly due to the activations of glial cells. Microglial cell is one of the important glial cells [2]. Neuroinflammation starts to occur at the early stage of AD [3]. Following activated, microglial cells release different kinds of proinflammatory signal molecules, including cytokines, growth factors, complement molecules, and chemokines [3]. Usually, under the conditions of microglial cells activation, the release of the inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 increase, and the release of the anti-inflammatory cytokine (IL-10) decreases [4]. The abnormal changes of these inflammatory and anti-inflammatory cytokines affect the neurophysiologic mechanisms regarding cognition and memory [5]. Indeed, these activated glial cells revolve around senile plaques and NFTs in the AD brain, suggesting that Aβ deposition could be a potent trigger of glial activation in the AD brain. We hypothesize that compounds inhibiting Aβ deposition may be able to attenuate the neuroinflammation in the central nervous system.

Many *in vitro* and *in vivo* models have been established to investigate the protective effects of small molecules against neuroinflammation.

Abbreviations: Aβ, Amyloid beta; AD, Alzheimer's disease; ANOVA, Analysis of variance; BBB, Blood-brain barrier; BCA, Bicinchoninic acid; BSA, Bovine serum albumin; COX-2, Cyclooxygenase-2; DAPI, 4′, 6-Diamidine-2′-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle medium; DMSO, Dimethyl sulfoxide; ECL, Electrochemiluminescence; EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol tetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GSK-3β, Glycogen synthase kinase-3β; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; HRP, Horseradish peroxidase; IL-1β, Interleukin-1β; IL-6, Interleukin-6; IL-10, Interleukin-10; iNOS, Inducible nitric oxide synthase; NFTs, Neurofibrillary tangles; NO, Nitric oxide; NP-40, Nonyl phenoxypolyethoxylethanol-40; PBS, Phosphate-buffered saline; PMSF, Phenylmethanesulfonyl fluoride; PVDF, Polyvinylidene difluoride; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, Standard error mean; TBS, Tris-buffered saline; (TNF)-α, Tumor necrosis factor-α

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Among these models, Aβ-induced neuroinflammation in BV-2 cell model has been widely used. Zhang et al used the Aβ-induced neuroinflammation in BV-2 cell model to evaluate the protective effect of hydroxy-safflor yellow A (HSYA), and found that HSYA inhibited Aβ 1-42 -induced inflammation and conferred neuroprotection partially through JAK2/STAT3 pathway [6]. With regard to *in vivo* models, the triple transgenic mouse model (3xTg-AD) is one of the popular models, which was developed by simultaneously inserting Swedish APP mutation and the P301 L tau mutation into PS1 mice [7]. In addition to significant NFTs and Aβ deposition, 3xTg-AD mice also display significant neuroinflammation. Sawmiller and colleagues reported that diosmin could reduce the neuroinflammation in the 3xTg-AD mice [8]. In the present study, we seek to evaluate the protective effect of an Aβ aggregation inhibitor against neuroinflammation in BV-2 cells stimulated by Aβ and in 3xTg-AD mice.

SLOH is a carbazole-based small molecule which has been shown to bind to Aβ *in vitro* and inhibited its aggregation [9]. SLOH can go through the blood-brain barrier (BBB) of an AD mouse brain at 1 mg/kg [9]. SLM is an analogue of SLOH, which has recently been reported to down-regulate the activity of glycogen synthase kinase-3β (GSK-3β) resulting in alleviating the cognitive impairments in a 3xTg-AD mouse model, and ameliorating the tau phosphorylation in okadaic acid-induced neuroblastoma SH-SY5Y cell model *via* down-regulating the activity of GSK-3β [10,11]. GSK-3β has been considered as an important therapeutic target that is involved in many neurodegenerative diseases [12]. Moreover, GSK-3β has been demonstrated as a modulator of inflammatory and anti-inflammatory cytokine levels in the brain, such as IL-1β, TNF-α and IL-10 [13].

In our recent study, SLOH has been shown to attenuate the cognitive deficits of 3xTg-AD mice *via* the reduction of Aβ burden, tau hyperphosphorylation and GSK-3β activity as well as neuroinflammation in 3xTg-AD mouse brains [14]. Albeit we initially observed that SLOH downregulated the expression of ionized calcium-binding adapter molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) in mouse brains [14], the interplay between different pro- and anti-inflammatory cytokines has not been studied. It would be of great interest to study the effects of SLOH on these cytokines, such as TNF-α, IL-1β, COX-2, iNOS and IL-10, in *in vitro* and *in vivo* systems to gain mechanistic insights into their roles in neuroinflammation. In this work, we seek to investigate the associated mechanisms of SLOH with regard to neuroinflammation in BV-2 cells stimulated by Aβ and in 3xTg-AD mice. We found that SLOH ameliorated neuroinflammation by modulating the pro- and anti-imflammatory cytokines and inhibiting the activation of Iba1 *in vivo via* down-regulating the activity of GSK-3β.

2. Experimental procedures

2.1. Cells

The immortalized mouse BV2 microglial cell line (BV-2) was a gift from Prof. Simon Lee, Institute of Chinese Medical Sciences, University of Macau.

2.2. Animal

The homozygous 3xTg-AD mouse (34,830) harboring PS1M146 V, APPSwe, and tauP301 L transgenes was purchased from the Jackson Laboratory and maintained in the Animal Facility of University of Macau. All procedures involving animals were approved by the University of Macau Animal Ethics Committee (protocol no.: UMAEC-13-2015). The housing conditions were controlled (temperature 23 °C; light from 7:00 to 19:00; humidity 60–65%), and food and water were freely available.

2.3. Reagents and chemicals

SLOH was synthesized as previously described [9] by a contract research organization (Chengdu DiRenTain Medical Technology Limited Company, Sichuan, PRC). Hexafluoro-2-propanol (HFIP, 105228A) and dimethyl sulfoxide (DMSO) were purchased from Sigma. SB216763 (S1075) was obtained from Selleckchem. Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. DAPI (D3571) and goat anti-rabbit secondary antibody, Alexa fluor® 488 (A11008) were purchased from ThermoFisher. The ECL reagent (6883), GSK-3β antibody (9315), phospho-GSK-3β (Ser9) antibody (9323), TNF-α (6945), IL-1β antibody (12703), IL-10 antibody (D13A11), COX-2 antibody (12282), iNOS antibody (13120), goat anti-rabbit secondary antibody (7074) and goat anti-mouse secondary antibody (7076) were purchased from Cell Signaling Technology. Anti-phospho-GSK-3β (Tyr216) (NB100-81946) was obtained from Novus Biologicals. GAPDH antibody (MAB374) was obtained from Millipore. Iba1 antibody (019-19741) was obtained from Wako. The primers for RT-PCR reactions were synthesized by IGE Biotechnology. LTD (Guangzhou, China). High pure RNA isolation kit (11828665001), transcriptor first Strand cDNA synthesis kit (4897030001), FastStart universal SYBR green master (4913914001), protease inhibitor cocktail (05892791001) and phosphatase inhibitor cocktail (04906845001) were purchased from Roche Life Science (Branford, USA). TNF-α Elisa kit (CSB-E04741 m) and nitric oxide Elisa kit (CSB-E14946 m) were purchased from Cusabio Biotech Co. Ltd (Wuhan, China).

2.4. Cell culture

BV-2 cells (passage 8 ~ 15) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air.

2.5. Aβ42 peptide and SLOH solution preparation

Aβ42 peptide oligomerization was carried out as previously reported [15]. The product from the Aβ42 peptide oligomerization was analyzed using SDS gel electrophoresis and stained with coomassie bright blue.

SLOH was dissolved in DMSO to form 10 mM stock solution, and then diluted into suitable concentration using phosphate buffer saline (PBS).

2.6. Measurement of nitrite and cytokines levels

BV-2 cells (2.5×10^5 per well) were seeded into 24-well plates and incubated for 24 h. Afterward, Aβ42 oligomer (5 μM) and SLOH (0, 0.3, 1 and 3 μM) were added to the cells and incubated for another 24 h. Later, the supernatants of the cultured cells were collected, the accumulated nitric oxide (NO) and TNF-α were measured using Elise kits according to the procedure recommended by the suppliers.

2.7. Animal treatment

3xTg-AD mice (10-month old, female n = 20) were divided into two groups randomly. SLOH was dissolved using 2% DMSO in PBS, and 2% DMSO in PBS was used as vehicle control. Group I: 3xTg-vehicle control and Group II: 3xTg-SLOH ($2\ mg\ kg^{-1}$). The $2\ mg\ kg^{-1}$ dose was selected based on behavior assessment results obtained from our previous study [14]. These mice were dosed with corresponding solutions five times a week (once daily from Monday to Friday) by intraperitoneal injection for one month.

2.8. Tissue preparation

At the end of the treatment, mice were anesthetized by means of