Tetrahydroxystilbene glycoside antagonizes β-amyloid-induced inflammatory injury in microglia cells by regulating PU.1 expression

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Inhibiting β-amyloid (Aβ)-induced microglial activation is proposed as an effective strategy for the treatment of Alzheimer’s disease. Tetrahydroxystilbene glycoside (TSG) is the main active ingredient of Polygonum multiflorum and has a wide range of biological properties, including anti-inflammation. Here, we focused on the function and regulatory mechanism of TSG in Aβ-induced N9 and BV2 cells. The results showed that Aβ treatment induced the activation of microglia cells and the production of inflammatory molecules, including inducible nitric oxide synthase, nitric oxide, cyclooxygenase 2, and prostaglandin E2, which were significantly inhibited by TSG pretreatment. Furthermore, we found Aβ exposure increased the levels of microglial M1 markers, interleukin (IL)-1β, IL-6, and tumor necrosis factor α, and the pretreatment of TSG suppressed the increase of M1 markers and enhanced the levels of M2 markers, including IL-10, brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, and arginase-1. PU.1 overexpression was found to eradicate the anti-inflammatory effects of TSG in Aβ-induced microglial cells.

Introduction

β-Amyloid (Aβ) deposition has been proposed as a crucial pathogenic event in the progression of Alzheimer’s disease (AD) [1]. Increasing evidence indicates that innate immunity may play an important role in the disorder, a factor mainly represented by microglial cells [2]. Under ordinary conditions, microglia are in an inactive state and they repeatedly monitor the neighboring environment for any changes to maintain the homeostasis of the brain. When microglia are activated by proinflammatory stimuli, they can repair brain lesions by phagocytosis or secretion of various inflammatory mediators; however, these inflammatory mediators may be harmful when microglia are continuously activated [3]. As Aβ level is increased in AD, microglia are overstimulated and thus produce inflammatory cytokines. Inhibiting aspects of Aβ-induced microglial activation is considered to be an effective strategy for AD treatment.

Tetrahydroxystilbene glucoside (2,3,5,4-tetrahydroxystilbene-2-glucoside, TSG) is the main active ingredient from the root of Polygonum multiflorum, which has a wide range of biological functions based on its antioxidant [4], anti-inflammatory [5], antidepressant [6], antiatherosclerotic [7], and antiaging [8] properties. Recently, many investigations have revealed that TSG is effective in the treatment of AD [9,10]. However, the functional roles and the molecular mechanisms of TSG in AD are as yet unclear. We aimed to investigate the effects of TSG on the microglial activation induced by Aβ in this study.

Here, we pretreated human N9 or BV2 microglial cell lines with TSG, and then exposed them to Aβ1-42. We found that TSG pretreatment successfully attenuated Aβ1-42-induced microglial activation and polarized towards M2 phenotype in these cells. The protective effects of TSG may be attributable to the regulation of PU.1 signaling.

Materials and methods

Cell culture

The mouse N9 and BV2 microglial cells were cultured in Dulbecco’s Modified Eagle’s medium containing 5% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C.

Construction of vector and cell transfection

Full-length Spi1 (protein name: PU.1; gene name: Spi1) cDNA sequence was amplified by PCR from cDNA template, which were obtained from N9 cells. The 5’-GCTGGATGTTACAGGCGTGCAAAATG-3’ and

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5′-CCGGGCGAGGGCTTAATGCTATGGCC-3′ are used as primer sequences as previously described [11]. The Spi1 cDNA fragments were cloned into pcDNA3.1 vector, named pcDNA3.1-PU.1. The empty pcDNA3.1 vector was used as a negative control. Microglial cells were seeded into six-well plates or 96-well plates 24 h before transfection, and then transiently transfected with pcDNA3.1-PU.1 or empty vectors using lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions.

**MTT assay**

The cell viability was assessed by MTT assay (Millipore, Boston, Massachusetts, USA) according to the manufacturer’s protocol. In summary, 10 µl of MTT solution was added to the different treated microglial cells and then incubated for 4 h. The absorbance was determined at 570 nm with a microplate reader (Wallace; PerkinElmer, Waltham, Massachusetts, USA).

**Western blot**

The total protein samples were lysed with the RIPA buffer (Sigma-Aldrich; St. Louis, Missouri, USA). The protein concentration was determined using a BCA Protein Assay Kit (Pierce; Rockford, Illinois, USA). Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore). After blocking in 5% skim milk at room temperature for 1 h, the membranes were probed with the anti-ionized calcium-binding adapter molecule (IBA) (1 : 200), anti-IL-10 (1 : 1000), anti-TNF-α, anti-IL-1β, anti-cyclooxygenase 2 (COX-2) (1 : 500), anti-interleukin (IL)-1α, anti-interleukin (IL)-1β (1 : 1000), anti-IL-6 (1 : 1000), anti-IL-10 (1 : 1000), anti-arginase-1 (Arg-1) (1 : 500), anti-inducible nitric oxide synthase (iNOS) (1 : 500), anti-glutathione-s-transferase (GST) (1 : 1000), anti-GAPDH (1 : 1000) overnight at 4°C. The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Then the blots were visualized using an Enhanced Chemiluminescence Detection Kit (Pierce; Rockford, Illinois, USA) and normalized to GAPDH signals.

**Determination of nitric oxide production**

The level of nitric oxide (NO) in the cell culture media was measured using the Griess method. The culture medium of the different treated microglial cells was removed and mixed with an equal volume of Griess reagent. The NO concentration was determined by the absorbance at 540 nm and compared with a standard curve of sodium nitrite absorbance.

**Measurement of prostaglandin E2**

The level of prostaglandin E2 (PGE2) in the cell culture media was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer’s protocol. The PGE2 concentration was determined by the absorbance at 490 nm.

**Enzyme-linked immunosorbent assay**

The supernatants of the different treated microglial cells were collected, and the concentrations of IL-1β, IL-6, IL-10, TNF-α, brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) were determined using corresponding ELISA kits according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analysis was carried out using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA). One-way analysis of variance was performed followed by the Bonferroni test for multiple group comparisons. Data are presented as the mean ± SEM. P values less than 0.05 were considered statistically significant.

**Results**

**Tetrahydroxystilbene glycoside suppresses β-amyloid-induced microglia cell activation in a concentration-dependent manner**

To assess the cytotoxicity of TSG to the microglia cells, we treated mouse N9 and BV2 cells with increasing concentrations of TSG for 48 h, and performed MTT assay to measure the cell viability. As shown in Fig. 1a and b, the cell viability in TSG groups (5, 10, 30, 60, and 90 μM) exhibits no significant difference compared with that in control group, suggesting that 5–90 μM TSG treatment has no cytotoxicity in N9 and BV2 cells. To investigate the effects of TSG on the microglia cell activation induced by Aβ, N9, and BV2 cells were treated with different concentrations of TSG for 24 h, followed by Aβ treatment for another 24 h. Then western blot was performed to evaluate the protein expression of IBA-1, a microglial activation marker. As shown in Fig. 1c and d, compared with control group, Aβ treatment induced significantly elevated expression of IBA-1. Compared with the Aβ group, IBA-1 expression showed a clear reduction in the TSG + Aβ group as TSG concentrations increased. These results suggest that TSG suppresses Aβ-induced microglia cell activation in a concentration-dependent manner, and 90 μM TSG was used in the following experiments.

**Tetrahydroxystilbene glycoside suppresses β-amyloid-induced production of nitric oxide and prostaglandin E2 in microglia cells**

To investigate the effects of TSG on the production of Aβ-induced inflammatory molecules, N9 and BV2 cells were treated with Aβ or the combination of TSG and Aβ. Then western blot assay was performed to measure the protein expression of iNOS and COX-2. Compared with the control group, Aβ treatment significantly upregulated the expression of iNOS and COX-2, which were
markedly inhibited by the pretreatment of TSG (Fig. 2a and b). Furthermore, we assessed the levels of NO and PGE2. As shown in Fig. 2c and d, the pretreatment with TSG significantly reduced the Aβ-induced increased production of NO and PGE2. These results suggest that TSG suppresses Aβ-induced expression of iNOS and COX-2 and inhibits production of NO and PGE2 in microglia cells.

**Tetrahydroxystilbene glycoside suppressed β-amyloid-induced production of microglial M1 markers**

To investigate the effects of TSG on the microglial M1 state induced by Aβ, ELISA assay and western blot were performed to detect the expression levels of M1 markers, including IL-1β, IL-6, and TNF-α. First, the ELISA data showed that compared with control cells, the concentrations of IL-1β, IL-6, and TNF-α were significantly greater in the cells treated with Aβ alone, which showed an obvious reduction in the cells treated with the combination of TSG and Aβ (Fig. 3a and b). Similarly, the western blot assay also revealed that the Aβ-induced production of IL-1β, IL-6, and TNF-α was significantly inhibited by the pretreatment with TSG (Fig. 3c and d). These findings suggest that TSG suppresses Aβ-induced production of IL-1β, IL-6, and TNF-α and inhibits the proinflammatory M1 state in microglia cells.

**Tetrahydroxystilbene glycoside promoted the production of M2 markers in β-amyloid-induced microglia cells**

We further determined the effects of TSG on the anti-inflammatory M2 state in Aβ-induced microglia cells. We measured the expression levels of microglial M2 markers, including IL-10, BDNF, GDNF, and Arg-1 by ELISA and western blot assay. As shown in Fig. 4a and b, the concentrations of IL-10, BDNF, and GDNF did not show obvious changes between the Aβ group and the control group. However, the TSG pretreatment significantly elevated the levels of IL-10, BDNF, and GDNF in
Aβ-induced microglia cells. Furthermore, the protein expression of IL-10 and Arg-1 showed a significant increase when N9 and BV2 cells were treated with the combination of Aβ and TSG compared with the cells treated with Aβ alone (Fig. 4c and d). These data indicate that TSG promotes the production of IL-10, BDNF, GDNF, and Arg-1 and facilitates the anti-inflammatory M2 state in Aβ-induced microglia cells.
The anti-inflammatory effects of tetrahydroxystilbene glycoside in β-amyloid-induced microglia cells were partly mediated by PU.1

To study the molecular mechanism mediating the anti-inflammatory effects of TSG in Aβ-induced microglia cells, we focused on PU.1, a transcription factor that is essential for the regulation of the immune response. First, we detected the protein expression of PU.1 by western blot assay and found that compared with the control group, Aβ treatment significantly upregulated the protein expression of PU.1, which showed an obvious reduction when the cells were pretreated with TSG (Fig. 5a and b). These results suggest that PU.1 may be involved in the regulation of TSG in Aβ-induced microglia cells. To confirm this suggestion, we transfected N9 and BV2 cells with overexpression vector pcDNA3.1-PU.1 or the empty vector, followed by the treatment of TSG and Aβ. Western blot assay revealed that the downregulation of IBA-1, iNOS, and COX-2, and the upregulation of Arg-1 induced by the pretreatment of TSG were partly reversed by PU.1 overexpression (Fig. 5c and d). Furthermore, we found that PU.1 overexpression in part reversed the reduction of IL-1β, IL-6, and TNF-α, and the increase in IL-10, BDNF, and GDNF induced by the pretreatment with TSG (Fig. 6). These data indicate that the biological effects of TSG on microglia cell activation and inflammation induced by Aβ are partly mediated by PU.1.

Discussion

Aβ deposition-induced microglia activation has been found to be a crucial event in the pathology of AD [12]. Inhibiting aspects of microglial activation is believed to be an effective therapy for the treatment of AD. To investigate the effects of TSG on the microglia cell activation induced by Aβ, western blot was performed to evaluate the protein expression of IBA-1, and the results showed that Aβ treatment significantly elevated the expression of IBA-1, which showed an obvious reduction in a concentration-dependent manner when the cells were pretreated with TSG. These results suggest that TSG suppresses Aβ-induced microglia activation.

Aβ deposition activates microglia cells to secrete inflammatory molecules, such as COX-2 and iNOS, and long-term inflammation is neurotoxic, inducing neuron injury and even death [13]. PGE2 is one of the inflammatory products of COX-2. Elevated levels of NO produced by iNOS have been found in response to a wide variety of proinflammatory stimuli in AD [14]. In this study, we found that Aβ treatment significantly upregulated the expression of iNOS and COX-2, and elevated the production of NO and PGE2, which were markedly inhibited by the pretreatment with TSG in N9 and BV2 cells. These results suggest that TSG suppresses the production of inflammatory molecules induced by Aβ in microglia cells.

Microglia can be activated in a classic activated state (M1 state) or alternative activated state (M2 state) depending
on the microenvironment [15]. The M1 state is believed to be proinflammatory and the M2 state may produce anti-inflammatory cytokines and neurotrophins involved in tissue repair and remodeling [16]. Therefore, polarizing the microglia from M1 phenotype to the M2 phenotype may be effective in treating microglial activation-induced degenerative disorders. In the study, Aβ-induced increased levels of IL-1β, IL-6, and TNF-α showed an obvious
reduction in the cells treated with the combination of TSG and Aβ. Meanwhile, TSG pretreatment significantly elevated the levels of IL-10, BDNF, and GDNF in Aβ-induced microglia cells. These findings suggest that TSG inhibited the proinflammatory M1 phenotype and facilitated the anti-inflammatory M2 phenotype in Aβ-induced microglia cells.

PU.1 has been reported as a critical regulator within the hematopoietic system, and also has a great effect on the anti-inflammatory effects of TSG in Aβ-induced microglia cells. We further revealed that the PU.1 overexpression partly reversed the reduction of IBA-1 and NOS2 gene expression. This is consistent with our previous finding that PU.1 overexpression in microglia cells significantly decreased neuro inflammatory effects of TSG in Aβ-induced microglia cells. These findings suggest that TSG may be an effective drug, and PU.1 may be a new target for reducing Aβ-induced microglia inflammation in AD. In addition, except for M1/M2 phenotypes, activated microglia can also be defined by morphology, phagocytosis (phagocytic receptors including CD36, SRA, and RAGE), and marker expression (including CD11b and CD45) [24,25]. Therefore, to better understand the role of TSG in Aβ-induced microglial activation and inflammatory injury, more careful analysis of the aforementioned different activation states is necessary in the future.

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Conflicts of interest

There are no conflicts of interest.

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