

PEG-PEI/siROCK2 inhibits A β ₄₂-induced microglial inflammation via NLRP3/caspase 1 pathway

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Objectives There is an urgent need to develop therapeutic strategies to improve the treatment outcome of Alzheimer's disease. The treatment strategy of gene therapy mediated by nanocarrier systems brings new hope for the treatment of Alzheimer's disease. ROCK2 is involved in various pathological processes of Alzheimer's disease and may be a potential target for the treatment of Alzheimer's disease. Our previous study indicated that PEG-PEI/siROCK2 [polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA, (PPSR)] prevented A β ₄₂-induced neurotoxicity and showed a promising prospect for the treatment of Alzheimer's disease. However, whether PPSR has an effect on the microglial inflammation in Alzheimer's disease is still unclear.

Materials and methods 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was used to detect the cytotoxicity of PEG-PEI and PPSR in primary microglial cells. Real-time PCR and western blotting were used to assess the expression of ROCK2 and nucleotide oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3)/caspase 1 pathway in primary microglial cells. ELISA assay was used to measure the effect of PPSR on attenuating the lipopolysaccharide (LPS) + A β -induced increase in IL-1 β .

Results PEG-PEI concentration less than 20 μ g/ml and the N/P (molar ratio of PEG-PEI amino/siRNA phosphate) ratio of PPSR less than 50 showed no significant

cytotoxicity in primary microglia cells. PPSR could effectively inhibit the expression of ROCK2 in primary microglial cells. A further study revealed that PPSR attenuates the LPS+A β -induced increase in IL-1 β without affecting cell viability. In addition, we found that PPSR suppressed the A β -induced NLRP3/caspase 1 pathway in primary microglial cells.

Conclusion PPSR inhibits A β ₄₂-induced microglial inflammation via NLRP3/caspase 1 pathway. *NeuroReport* 33: 26–32 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Alzheimer's disease is the most frequent form of dementia in the elderly and is defined by the combined presence of amyloid β -protein (A β) and tau protein [1,2]. The number and proportion of those who are suffering from Alzheimer's disease continue to increase worldwide. By 2050, Alzheimer's disease is expected to affect 13.8 million adults [3]. However, at present, there is no successful therapy to stop or slow the progression of Alzheimer's disease. Thus much research has focused on the mechanisms and treatment strategies underlying Alzheimer's disease.

Inflammation plays a pivotal role in the pathogenesis of Alzheimer's disease [4]. Excessive neuroimmune inflammation damages neurons, which release a variety of toxic contents such as A β ₄₂. A disturbed balance between the production and degradation of A β can drive inflammatory processes in astrocytes and microglial cells and initiate a vicious circle [5], eventually leading to neuronal death and brain tissue atrophy. Therefore, the inflammation of microglia may be a promising treatment target for Alzheimer's disease.

Gene therapy has shown significant potential for the treatment of Alzheimer's disease in preclinical trials [6]. However, the challenge of gene therapy is well-tolerated and effective delivery vectors. Nanocarriers are promising gene therapy carriers for central nervous system diseases due to their biosafety and ability to cross the blood-brain barrier [7]. Polyethylenimine (PEI) is one of the most successful polycationic carriers and when engrafted by

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hydrophilic PEG has enhanced biocompatibility and efficiency when used for nonviral gene delivery [8]. At present, polyethyleneglycol-polyethyleneimine (PEG-PEI) vectors have been applied to Alzheimer's disease in our early experiments and achieved good results [9].

Rho-kinase, also known as ROCK, is widely recognized as a member of the serine/threonine-protein kinase family, which plays crucial roles in various cellular functions such as cell migration, proliferation and survival [10]. Aberrant activation of the Rho/ROCK pathway has already been noticed in multiple disorders of the central nervous system, including Alzheimer's disease [11]. Our previous studies have found that ROCK2 was a vital target gene for the therapy of Alzheimer's disease. *In vitro*, our studies showed that PEG-PEI/siROCK2 [polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA, (PPSR)] prevented A β_{42} -induced neurotoxicity [9]. *In vivo*, we found that intracranial injection of PPSR improved the cognitive impairments of senescence-accelerated mouse (SAM) [12]. Therefore, ROCK2 may be a crucial target gene for the therapy of Alzheimer's disease. But whether there is a relationship between ROCK2 and microglial inflammation in Alzheimer's disease is still unclear.

With these backgrounds, we synthesized PPSR to inhibit the expression of ROCK2 in primary microglial cells. Our study aims to evaluate the biologic properties and molecular mechanism of PPSR on A β_{42} -induced microglial inflammation. The results showed that PPSR inhibited A β_{42} -induced microglial inflammation via the nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3)/caspase 1 pathway, which provides evidence to support the therapeutic potential of PPSR in Alzheimer's disease.

Materials and methods

Cell culture and treatment

Primary microglial cells were cultured according to our previously published protocol [13]. Briefly, P1 C57BL/6J mice were stripped of meninges and minced in a hepes-balanced salt solution (Mediatech Inc., Herndon, Virginia, USA). All animals used for cell isolation were treated according to the legal and ethical requirements of the University of Sun Yat-sen University (Approval Number: IACUC-2021020801). Cells were dissociated and then cultured in F12/Dulbecco's Modified Eagle Media (DMEM) medium (Gibco, Invitrogen, Carlsbad, California, USA) containing 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen) at 10^4 cells/cm². The cell culture medium was replaced with fresh complete media every 2 days. Microglial cells were harvested by tapping the flasks and collecting the floating cells on day 12. Microglia were pelleted by centrifugation and resuspended in F12/DMEM with 10% fetal bovine serum and maintained at 37 °C under 5% CO₂. The next day, microglia were incubated with PPSR, PEG-PEI, siROCK2

or PBS for 24 h. The microglia were then primed with lipopolysaccharide (LPS) (50 ng/mL) for 3 h and treated with 2.5 μ M A β_{42} for 6 h in a serum-free DMEM containing 1% P/S.

Preparation of A β fibrils

A β_{42} oligomers were prepared as previously described [14]. Briefly, A β_{1-42} (Bachem, H-1368) dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 500 μ M and stored at -80 °C. To prepare A β fibrils, A β_{1-42} was diluted to a concentration of 50 μ M by adding DMEM-F12 with 10% FBS for 24 h at 37 °C. After preparation of A β fibrils, they were diluted with cell culture medium to 2.5 μ M. To confirm the fibrillization of A β , thioflavin S (Sigma, T1892) was added to the solution and then A β can be identified by an apple green color under a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). To analyze the images, a spectrofluorometer VICTOR X4 (PerkinElmer, Massachusetts, USA) was used at an excitation of 405 nm and an emission of 535 nm.

Preparation of polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA complexes

PEI (MW = 25 kD), monomethoxy PEG (mPEG-OH, MW = 2 kD), N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). PEG-PEI was synthesized internally using techniques previously reported in our laboratory [8]. The siROCK2 that targeted ROCK2 mRNA in mice was purchased from GenePharma (Shanghai, China). PPSR complexes were synthesized and characterized as described previously by our laboratory [8]. The amount of delivery agent (PEG-PEI) to complex the small interfering RNA (siROCK2) was based on various N/P (molar ratio of PEG-PEI amino/siRNA phosphate) ratios. The PPSR solution was vortexed for a short time and further incubated for 30 min at room temperature before use.

In-vitro transfection

For transfection experiments, the primary microglial cells were seeded in a 24-well plate at a density of 5×10^4 cells/well at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The original medium in each well was replaced with optiMEM (Invitrogen). PPSR complexes with N/P ratios of 50 were added to the cells and incubated for 6 h at 37 °C. After that, the medium was replaced with the same volume of fresh complete medium, for another 18-h incubation.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell metabolism assay was used to evaluate the cytotoxicity of PPSR complexes. Briefly, the primary microglial cells were seeded in a

96-well plate and treated according to the set conditions. After that, the cells were incubated with MTT reagent (0.5 mg/ml) for 4 h at 37 °C. Then, the MTT solution was removed and 100 μ L of DMSO was added to dissolve the resulting formazan crystals. The optical density (OD) was measured using a Synergy plate reader (BioTEK instruments, Winooski, Vermont, USA) at 570 nm with a reference wavelength of 630 nm. The relative cell viability rates was calculated as follows: Cell viability (%) = $(OD_{\text{sample}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100\%$.

ELISA

NLRP3 inflammasome activation by A β was assessed by measuring IL-1 β and tumor necrosis factor α (TNF α) secretion. The supernatants were centrifuged and harvested for ELISAs. Levels of IL-1 β (R&D Systems, MLB00C) and TNF α (BMS2034 from Invitrogen) in cell-cultured supernatants samples were measured using ELISA kits according to the manufacturers' instructions. The OD was analyzed at 450 nm photometrically with a microplate reader (Infinite M200; Tecan). The concentration of IL-1 β and TNF α was quantified using the relevant standard curves.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was synthesized according to the manufacturer's instructions using the PrimeScript RT Reagent Kit (Takara). The quantitative real-time PCR (qRT-PCR) reaction was performed by using the all in-One q-PCR Mix Kit (GeneCopoeia, AOPR-0200) in the LightCycler 480II PCR System (LightCycler 480II, Roche, USA). Differences between samples and controls in gene expression were quantified using the $2^{-\Delta\Delta C_t}$ method. Primers were provided as follows: ROCK2 forward, GCGATGCTGAGCCTGATGA and reverse, GCACAGGCAATGACAA -CCAT. β -actin forward, GTGACGTTGACATCCGTAAGA and reverse, CCCGGACTCATCGTACTCC.

Western blotting

The total amount of protein was extracted using cytosolic/nuclear protein lysis buffer (Beyotime, P0027). Protein concentrations were measured using the bicinchoninic acid assay kit (ThermoFisher Pierce) according to the manufacturer's instructions. For western blotting, protein lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were incubated with primary antibodies against ROCK2 (Cell Signaling Technology, 1:1000), NLRP3 (Cell Signaling Technology, 1:500), caspase-1 (Santa-Cruz Biotechnology, 1:50), IL-1 β (Santa-Cruz Biotechnology, 1:50), β -actin (Cell Signaling Technology, 1:1000) and glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology, 1:1000) at 4 °C overnight, followed by incubation with anti-rabbit IgG (MultiSciences, 1:5000) or anti-mouse IgG (MultiSciences, 1:5000) at

room temperature for 1 h. Protein bands were determined using an enhanced chemiluminescence western blot detection kit (KeyGEN BioTECH, KGP1126).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0 (IBM, Armonk, New York, USA). All experiments were performed in triplicate and each assay was repeated at least three times independently. Data are presented as mean \pm SD and *P* values <0.05 are considered statistically significant. Comparisons between two groups were analyzed with Student's *t*-test. Multiple comparisons of more than two groups were conducted with one-way analysis of variance followed by Bonferroni's post hoc test.

Results

Neurotoxicity of Polyethyleneglycol-polyethyleneimine and Polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA-induced in primary microglial cells

To verify the biosafety of PEG-PEI and PPSR, we first tested their cytotoxicity in primary microglial cells. As shown in Fig. 1a, the cytotoxicity of PEG-PEI increased with the increase of concentration. When PEG-PEI concentration exceeded 20 μ g/mL, the cell viability decreased significantly (*P* < 0.001, Figure. 1a). The cytotoxicity of PPSR complexes correspondingly increased with the increase of the N/P ratio. When the N/P ratio reached 50, the cell viability was 85.3% (Fig. 1b). Cell viability was remarkably decreased when the N/P ratio exceeded 50 (*P* < 0.001, Fig. 1b). These results indicated that a PEG-PEI concentration less than 20 μ g/mL and N/P ratio of PPSR less than 50 showed good biosafety in primary microglia cells.

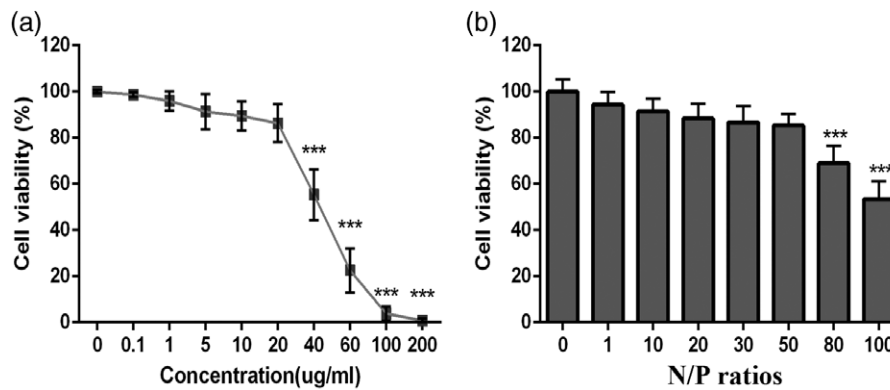
Gene-silencing effect of polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA in Primary microglial cells

The Gene-silencing effect of PPSR in primary microglial cells was evaluated at both the mRNA and protein levels of ROCK2. As shown in Fig. 2a, PPSR complexes significantly inhibited ROCK2 mRNA expression compared with the control group (*P* < 0.001, Fig. 2a). The protein expression of ROCK2 level was significantly decreased in the PPSR group compared with the control group (*P* < 0.001, Fig. 2b and c). These results indicated that PPSR effectively inhibits the expression of ROCK2 in primary microglial cells.

Polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA attenuates the lipopolysaccharide+A β -induced increase of IL-1 β in primary microglial cells without affecting cell viability

To investigate the effect of PPSR on A β -induced secretion of inflammatory cytokines in primary microglial cells, the levels of IL-1 β and TNF α were measured in the supernatant fractions of the microglia using ELISA.

Fig. 1



In vitro cytotoxicity of PEG-PEI and PPSR in primary microglial cells determined by an MTT assay. (a) Primary microglial cells treated with PEG-PEI at various concentrations from 0 to 200 μ g/ml. (b) Primary microglial cells treated with PPSR at various N/P ratios from 0 to 100. The dose of PPSR was 100 nM in each well and incubation time was 24 h. Values are mean \pm SD ($n=3$). *** $P<0.001$ vs. control. PPSR, polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA; .

The results revealed that LPS+A β (50 ng/ml LPS plus 2.5 μ M A β_{42}) significantly increased the expression of IL-1 β and TNF α ($P<0.001$, Fig. 3a and b). The level of IL-1 β was significantly reduced in the LPS+A β +PPSR group compared with the LPS+A β group ($P<0.001$, Fig. 3a and b). However, the level of TNF α showed no significant difference between the LPS+A β +PPSR group and the LPS+A β group ($P>0.05$, Fig. 3a and b). These results indicated that PPSR attenuates the LPS+A β -induced increase of IL-1 β in primary microglial cells. To test whether the treatment of the four groups affected the viability of primary microglial cells, the MTT assay was performed. There was no significant difference in cell viability among the groups ($P>0.05$, Fig. 3c). Together, these results indicated that PPSR attenuates the LPS+A β -induced increase of IL-1 β in primary microglial cells without affecting cell viability.

Polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA suppressed the A β -induced NLRP3/caspase 1 pathway in primary microglial cells

To explore the effect of PPSR on IL-1 β pathway activation, the expression of NLRP3, pro-caspase-1 and caspase-1 in each group of primary microglial cells was measured by western blotting. The protein expression levels of NLRP3, pro-caspase-1 and caspase-1 were significantly increased in the LPS+A β group compared with the control group ($P<0.01$, Fig. 4a-d). These results indicated that PPSR suppresses the A β -induced NLRP3/caspase 1 pathway in primary microglial cells.

Discussion and conclusion

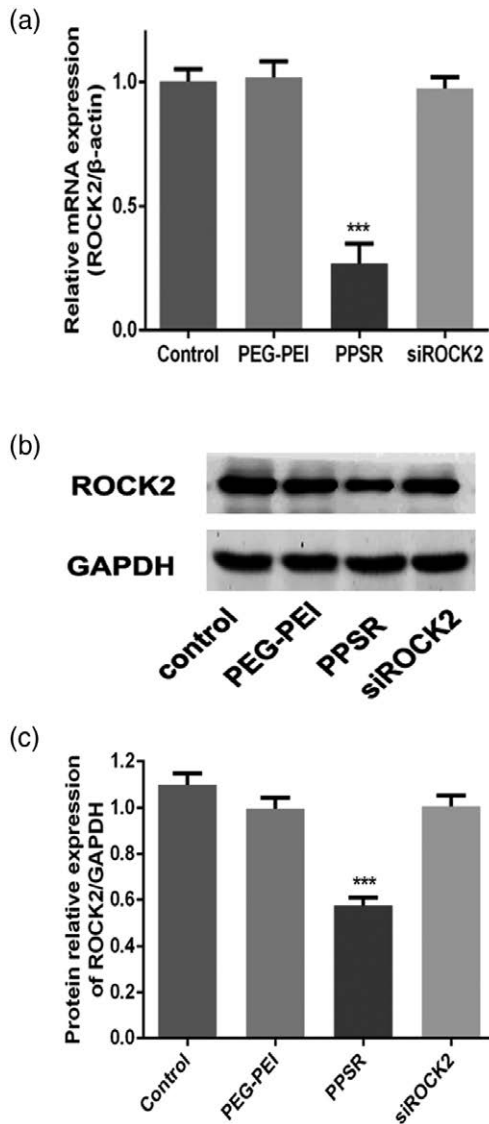
Since its discovery in 1996, ROCK has been shown to contribute to several physiological processes, especially in neurite growth and sprouting [15]. Inhibition of the Rho/ROCK pathway has proven efficacious in a

transgenic mouse model of Alzheimer's disease [16]. We have also demonstrated that the inactivation of the Rho/ROCK signaling pathway can alleviate the impairment of Alzheimer's disease [12]. To study the relationship between ROCK2 and microglial inflammation in Alzheimer's disease, we successfully synthesized the PPSR compound and confirmed that PPSR alleviated A β_{42} -induced microglial inflammation by targeting the NLRP3/caspase 1 pathway.

PEG-PEI copolymer has potential medical applications in drug and gene delivery [17,18]. Compared with viral vectors, polymer materials have advantages of nonimmunogenicity and high gene transfer efficiency. Based on previous experiments, we successfully synthesized PEG-PEI and PPSR. But there are still some concerns about the biosafety of the PEG-PEI copolymer, which are related to its concentration and N/P ratios. Our study showed that the toxicity increased with the concentration of PEG-PEI. These findings are consistent with other experimental studies showing that as the polymer concentration increased, the cytotoxicity gradually increased [19]. Our study also confirmed an increase in toxicity of PPSR with increasing N/P ratios, which is in accordance with other nanomaterials [20]. Increased N/P results in better transfection efficiency but with more potent cytotoxicity. Thus, to obtain lower cytotoxicity and higher transfection efficiency, PEG-PEI concentration less than 20 μ g/mL and the N/P ratio of PPSR less than 50 were selected to treat primary microglial cells *in vitro*. Our experiment finally confirmed that the PPSR at N/P of 50 effectively inhibited the expression of ROCK2, which was consistent with our previous study [8].

Previous studies have confirmed that the excessive activation of microglia resulted in significant changes

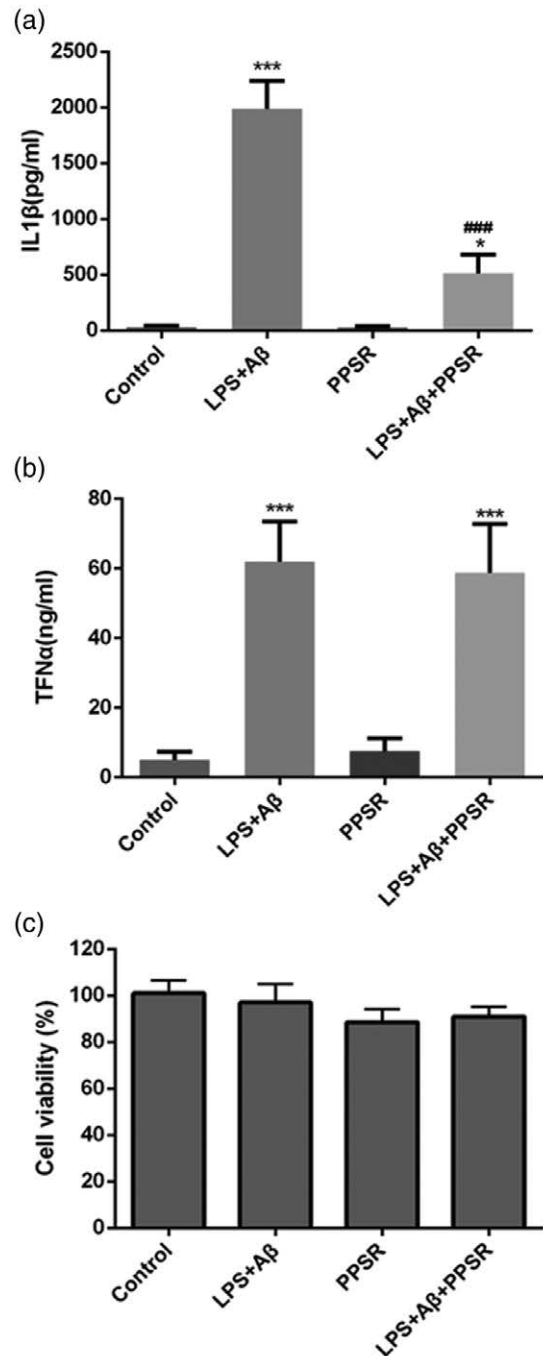
Fig. 2



Gene-silencing effect of PPSR in primary microglial cells. (a) The gene-silencing effect of PPSR (100 nM) in primary microglial cells was detected by RT-PCR. (b) The gene-silencing effect of PPSR (100 nM) in primary microglial cells was detected by western blotting. (c) The relative protein expression levels of ROCK2 in each group. The control was treated with PBS. Values are mean \pm SD, *** P <0.001, compared with the control. PPSR, polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA; RT-PCR, reverse transcription-PCR

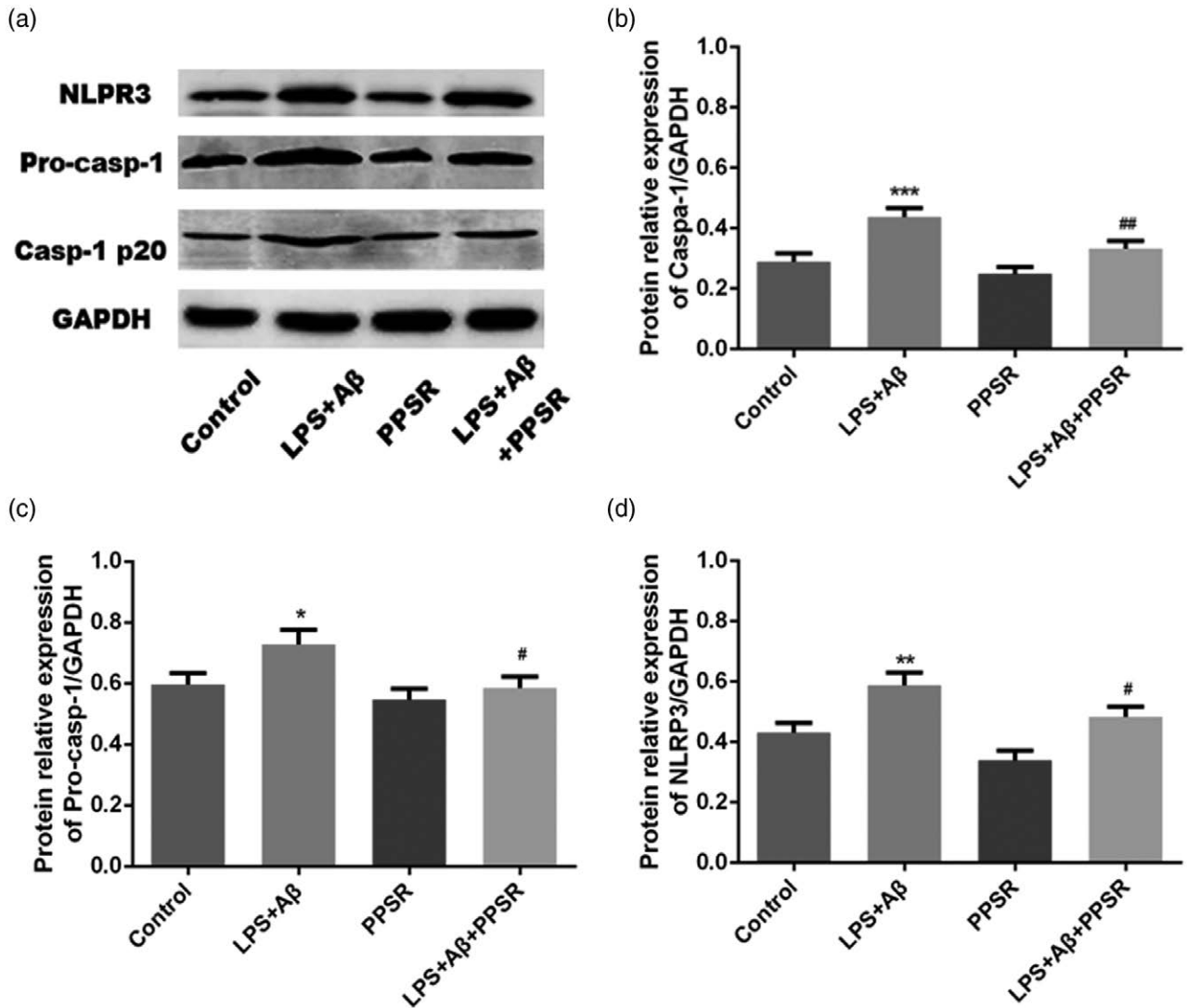
in its structure and function, releasing massive neuroimmune inflammatory factors such as TNF- α and IL-1 β [21]. Since A β_{42} oligomers cannot directly lead to the production of TNF- α and IL-1 β in microglia, while LPS combined with A β_{42} oligomers can significantly increase the secretion of TNF- α and IL-1 β [22], we used LPS+A β oligomers to simulate microglia cells as the cell model of Alzheimer's disease. Our results demonstrated that LPS+A β pretreatment could

Fig. 3



PPSR attenuates the LPS + A β -induced increase in IL-1 β without affecting cell viability. (a) The IL-1 β level was measured in the supernatants of the microglia 24 h later using ELISA. (b) The levels of TNF α were measured in the supernatant fractions of the microglia 24 h later using ELISA. Primary microglial cells were transfected with the indicated PPSR and preactivated with LPS (50 ng/mL) for 3 h before the A β fibrils (2.5 μ M) were added. (c) In-vitro cytotoxicity of four groups in primary microglial cells was determined by an MTT assay. The control was treated with PBS. Values are mean \pm SD, *** P <0.001, compared with the control. PPSR, polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA; IL, interleukin; LPS, lipopolysaccharide; TNF α , tumor necrosis factor α

Fig. 4



PPSR suppressed A β -induced NLRP3/caspase 1 pathway response Primary microglial cells. (a) Western blot analysis of inflammasome complex (NLRP3, pro-CASP1 and CASP1 p20). (b-d) The relative protein expression levels of NLRP3, pro-CASP1 and CASP1 p20 in each group. The control was treated with PBS. Values are mean \pm SD, *** P <0.001, compared with the control. PPSR, polyethyleneglycol-polyethyleneimine deliver ROCK2-siRNA; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family pyrin domain containing 3; NOD, nucleotide oligomerization domain.

aggravate the inflammatory response in primary microglial cells, causing increased release of inflammatory factors such as IL-1 β and TNF α , which were consistent with previous studies [23]. Our results also revealed that PPSR could reduce the LPS+A β -induced increase of IL-1 β in primary microglial cells, but did not affect the increased production of TNF α . These findings suggested that ROCK2 does not completely ameliorate the microglial cell inflammation induced by LPS+A β , but specifically inhibits the IL-1 β inflammatory pathway. However, the precise mechanism underlying the role of ROCK2 and IL-1 β in primary microglial cells remains unclear.

The inflammasome, a multiprotein complex, specifically cleaves proinflammatory cytokine precursors into activated proinflammatory cytokines that mature and are secreted, leading to widespread inflammation in the brain [24]. Recently, attention has turned to the NLRP3 inflammasome. The core of the NLRP3 inflammasome is the NOD receptor family of NLRP3 proteins. Therapies targeted specifically to the NLRP3 inflammasome could relieve the dysfunctions following Alzheimer's disease [25]. A β_{42} is reported to activate the NLRP3 inflammasome in microglial cells, leading to the production of proinflammatory cytokines and inflammation [26], which is consistent with our findings. Researchers proved that

inhibiting the NLRP3 inflammasome promotes clearance of amyloid- β and cognitive function in APP/PS1 mice [27]. Therefore, we wanted to explore whether PPSR affects IL-1 β through the NLRP3 pathway.

At present, there are few studies on the relationship between ROCK and NLRP3. Previous studies revealed that the inhibition of ROCK2 significantly reduced the expression of NLRP3 and therefore suppressed the inflammatory response induced by NLRP3 [28]. Our results showed that inhibiting ROCK2 significantly reduced the expression of NLRP3, which was consistent with the previous reports. The increase and activation of NLRP3 mediate the activation of the NLRP3/caspase1 pathway and the release of IL-18/IL-1 β [29]. A β deposition leads to an increase in IL-1 β , which is produced in a biologically inactive form and requires caspase-1 activation and secretion [30]. Therefore, according to the above studies, we speculated that PPSR could inhibit IL-1 β by suppressing the expression of NLRP3.

In summary, our study demonstrated that PPSR inhibits A β ₄₂-induced microglial inflammation via NLRP3/caspase1 pathway. But, how PPSR regulates the NLRP3/caspase1 pathway and its specific mechanism are still unclear, which need further study. Overall, this study revealed a new mechanism of Alzheimer's disease, indicating that PPSR is a potent therapeutic strategy for Alzheimer's disease.

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Conflicts of interest

There are no conflicts of interest.

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