Abstract. Alzheimer's disease (AD) is a chronic and irreversible neurodegenerative disorder. Abnormal aggregation of the neurotoxic amyloid-β (Aβ) peptide is an early event in AD. The activation of astrocytic α7 nicotinic acetylcholine receptor (α7 nAChR) can inhibit Aβ aggregation; thus, the molecular mechanism between α7 nAChR activation and Aβ aggregation warrants further investigation. In the present study, Aβ oligomer levels were assessed in astrocytic cell lysates after treatment with Pnu282987 (a potent agonist of α7 nAChRs) or co-treatment with LY294002, a p-Akt inhibitor. The levels of heat shock factor-1 (HSF-1), heat shock protein 70 (HSP-70), and αB-crystallin (cryab) in astrocytes treated with Pnu282987 at various time-points or co-treated with methyllycaconitine (MLA), a selective α7 nAChR antagonist, as well as co-incubated with LY294002 were determined by western blotting. HSF-1 Kd in astrocytes effectively decreased cryab, but not HSP-70 expression. HSF-1 is necessary for the upregulation of cryab expression, but not for that of HSP-70. HSF-1 and HSP-70 have a neuroprotective effect. Furthermore, the neuroprotective effect of Pnu282987 against Aβ aggregation was mediated by the canonical PI3K/Akt signaling pathway activation.

Introduction

Alzheimer's disease (AD), a chronic but irreversible neurodegenerative disorder with the highest incidence of age-related dementia, is mainly caused by abnormal aggregation of the neurotoxic amyloid-β (Aβ) peptide, a product that forms following proteolysis of the amyloid precursor protein (APP) (1). Pathological characteristics of AD often include neuronal apoptosis, synaptic loss and cognitive impairment, which are considered to be associated with abnormal aggregation of Aβ (2,3). Furthermore, it has been revealed that increased Aβ formation results in high levels of reactive oxygen species and an increased activation of proinflammatory cytokines, associated with neuroinflammation in AD (4). Because abnormal accumulation of Aβ plays a key role in the progression of AD, understanding how to inhibit its aggregation warrants further study.

Astrocytes can provide energy to neurons through the astrocyte-neuron shuttle (5), thereby, affecting their metabolism and synaptic activity, which is known to be important for memory formation (6,7). Notably, Aβ can promote the expression of inflammatory factors and inhibit the activity of Aβ-cleaving serine proteases in astrocytes (8,9), which is associated with AD severity (10).
The α7 nicotinic acetylcholine receptors (nAChRs) are pentameric molecules that belong to the ligand-gated ion channel family involved in regulating the permeability of Ca2+, associated with neuroprotective and anti-inflammatory effects in the AD brain (11). α7 nAChRs are abundantly expressed in the central and peripheral nervous system, and spinal cord (12). α7 nAChRs can form a stable complex with Aβ in neuritic plaques and neurons that are associated with the pathogenesis of AD, which results in attenuation of Aβ neurotoxicity (13). The expression of α7 nAChRs is significantly increased in the cerebral cortex and hippocampus of patients with AD, which is closely associated with Aβ deposits (14,15). However, it is not clear whether the physiological functions of α7 nAChRs are related to Aβ aggregation and deposition in astrocytes.

Heat shock proteins (HSPs), a conservative protein family induced by heat shock and heavy metals, among others, are widely expressed in prokaryotes and eukaryotes (3). HSPs, such as HSP-70 and αB-crystallin (Cryab), can effectively suppress the aberrant accumulation of Aβs (16). Moreover, Cryab combines with Aβs, significantly inhibiting their accumulation and neurotoxicity (17,18). Heat shock factor 1 (HSF-1) is activated through multiple steps; it then translocates to the nucleus, where it induces HSPs in response to stress by binding to the heat shock element (HSE). HSF-1 protects neurons from death by regulating the expression of HSPs (19). However, it is still not known whether this protective effect is mediated by α7 nAChRs stimulation.

In the present study, it was reported that PNU282987, a potent agonist of α7 nAChRs, can significantly enhance suppression of Aβ aberrant accumulation via the upregulation of endogenous Cryab and HSP-70 in cultured astrocytes. In fact, it was demonstrated that HSF-1 expression was necessary for the upregulation of Cryab, but not for that of HSP-70. Furthermore, the neuroprotective effect of PNU282987 against Aβ aggregation was mediated via activation of the canonical PI3K/Akt signaling pathway after α7 nAChRs.

Materials and methods

Materials. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco; Thermo Fisher Scientific, Inc. Synthetic drugs included Aβ$_{1-42}$, PNU282987, dimethylsulfoxide (DMSO) and methyllycaconitine (MLA) (Merck KGaA). The following antibodies were used in the present study: Mouse polyclonal anti-Aβ, 1-16 (cat. no. 803015; BioLegend, Inc.); rabbit polyclonal anti-HSP-70 (cat. no. AB9920; Sigma-Aldrich; Merck KGaA), anti-Cryab (cat. no. ab13496; Abcam), anti-β-actin (cat. no. sc-47778; Santa Cruz Technology, Inc.); rabbit monoclonal anti-phospho-(cat. no. 7252C) and non-phospho-specific Akt (cat. no. 2920) and anti-HSF-1 (cat. no. 12972; Cell Signaling Technology, Inc.), and peroxi-dase-conjugated secondary antibodies (cat. no. sc-2357; Santa Cruz Technology, Inc.). Phosphate-buffered saline (PBS), trypsin and penicillin-streptomycin were obtained from HyClone; GE Healthcare Life Sciences, Lipofectamine® 2000 was purchased from Invitrogen; Thermo Fisher Scientific, Inc., and the LY294002 inhibitor was obtained from Cell Signaling Technology, Inc. Culture flasks were purchased from Corning, Inc. The enhanced chemiluminescence system (ECL) was obtained from Amersham; GE Healthcare, and polyvinylidene fluoride (PVDF) membranes were purchased from EMD Millipore. Sprague-Dawley (SD) pregnant rats were purchased from the Animal Experiment Center of Guizhou Medical University (GuiZhou, China).

Primary astrocyte cultures. All animal experiments in the present study were approved by the Animal Care Committee of Guizhou Medical University and were implemented in strict accordance with the relevant guidelines (no. 1503008). A total of 20 SD rats (8 weeks old, weight 240-280 g) were randomly divided into two groups, 10 (5 males and 5 females) in each group. Rats had access to food and water ad libitum and were maintained at 25°C (relative humidity 65%) with a 12-h light/dark cycle. Astrocytes were separated from the cerebral cortex of newborn SD rats according to a previously described method (20). Briefly, the cerebral cortex was separated and cut, a 10X volume of trypsin digestion buffer was added for 15 min at 37°C. Samples were centrifuged at 1006.2 x g, 25°C for 5 min in complete medium (DMEM with 10% FBS and 1% penicillin-streptomycin). Cells were then resuspended with complete medium and transferred to 25-cm$^2$ culture flasks for ~9 days. Cells were then purified by centrifugal methods (83.8 x g, 37°C, 6 h). Purified cells were sub-cultured in 25 cm$^2$ culture flasks (37°C, 5% CO$_2$). In this way, ~95% of the astrocytes could be purified, and they were identified using immunostaining with rabbit anti-GFAP antibody (1:200) and anti-rabbit IgG Cy3 (1:200). Rats were placed into enclosed flow cages for 5 min, and then 100% CO$_2$ was infused at a 30% volume per minute displacement, in order to euthanize the animals. Subsequently, rats were rendered unconscious by CO$_2$ inhalation (confirmed by slow deep breathing and absence of response to toe-pinch).

Preparation of Aβ oligomers. Aβ oligomers were prepared according to a previous method (21). Briefly, Aβ peptides were dissolved in hexafluoroorisopropanol (HFIP) at a concentration of 1 mM. After evaporating HFIP, the peptide membrane was stored at -80°C. Aβ peptides were dissolved with DMSO and diluted to 1 μm with DMEM under sterile conditions. The quality of Aβ oligomers was identified by 12 alkyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (22).

Treatment of astrocytes. To evaluate the role of PNU282987 in HSP regulation, cultured astrocytes were treated with PNU282987 (5 μM) at different time-points (6, 12, 18, 24 h) in an incubator at 37°C. The regulatory effect of PNU282987 was partly inhibited by MLA (100 nM,), an α7 nAChR antagonist, at 37°C for 2 h. The concentration of MLA used was based on previous studies (23,24). To explore whether the regulatory effect of PNU282987 was mediated by the PI3K signaling pathway, astrocytes were pretreated with LY294002 (10 μM) for 2 h at 37°C before PNU282987 treatment. The concentration of LY294002 was based on previous studies (19,20). The medium was replaced by DMEM containing PNU282987 (5 μM), and the astrocytic cultures were incubated for 18 h at 37°C. After removing the medium, astrocytes were further exposed to DMEM containing Aβ oligomers (1 μM) for 24 h at 37°C.
Western blot analysis. Cells were washed three times with pre-chilled PBS in a six-well plate; then, cells were incubated on ice for 3 h with 60 µl/well of RIPA buffer containing protease and phosphatase inhibitors (cat. no. 98065; Cell Signaling Technology, Inc.). The supernatant was collected after centrifugation at 4℃ for 15 min at 4,024.8 x g, and the concentration of the supernatant protein was assessed with a BCA assay (Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions. Proteins were loaded (10 µg/lane) and resolved in 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, and then blocked with 5% non-fat milk at room temperature (RT) for 2 h. Membranes were washed three times with TBS buffer with 0.1% Tween-20 (TBST); subsequently, the proteins were incubated with various primary antibodies overnight at 4℃. Antibodies included: Anti-phospho-Akt (Ser 473; 1:1,000); anti-HSF-1 (1:1,000); anti-CRYAB (1:1,000); anti-β-Amyloid 1-42 (1:1,000); anti-HSP-70 (1:1,000); or anti-non-phospho-Akt (1:1,000). Following the overnight incubation, the membranes were rinsed three times with TBST (5 min each) and proteins were incubated with the appropriate secondary antibody for 1 h at RT. GAPDH (1:1,000; cat. no. MA5-157381; Thermo Fisher Scientific, Inc.) or β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) were used as internal controls. Protein bands were detected with an ECL kit, according to the manufacturer’s instructions. Quantiﬁcation was performed using ImageJ software (version 1.46; National Institutes of Health).

Transfections. Cell transfections were carried out according to previously described protocols (25,26). Briefly, cells were cultured in six-well plates for 24 h at a density of 5x10⁵ cells and allowed to adhere for 24 h. The target sequence of the rat HSF-1 shRNA plasmid (Qiagen, Inc.) was 5′-TGTCCTAA CAAGCATCATTCAATT-3′. Scrambled shRNA (Qiagen, Inc.) or HSF-1-specific shRNA (2 µg), plus reagents (2 µl), and Lipofectamine 2000 (6 µl) were mixed with Opti-MEM medium (500 µl; cat. no. 31985062; Thermo Fisher Scientific, Inc.) for 20 min. Cells were incubated at 37℃ for 48 h in this medium containing shRNA. Subsequently, the cells were washed 3 times with PBS and fresh medium was added. The transfected cells were seeded in the selection medium (containing Hygromycin 340 µg/ml) for 12 days. Well-separated antibiotic resistant clone cells were identiﬁed and expanded for subsequent experiments. The identiﬁed cells were treated with PNU (5 µM) for 18 h at 37℃. The time interval between transfection and subsequent experimentation was 15 days.

Statistical analysis. To compare differences among groups, one-way ANOVA was used with Tukey’s post hoc test for multiple comparisons. Data results were analyzed with SPSS for statistical analysis. Data are presented as the mean ± SD; each experiment was repeated independently three times. *P<0.05 vs. the αβ group; P<0.01 vs. the control group. αβ, amyloid-β; PNU, PNU282987.

Results

PNU282987 can enhance astrocytes to inhibit Aβ aggregation. Pretreatment with 5 µM of the potent agonist of α7 nAChRs PNU282987 for 18 h followed by treatment with 1 µM Aβ1-42 for 24 h signiﬁcantly inhibited Aβ accumulation, indicating that PNU282987 enhanced astrocytes to inhibit Aβ aggregation (Fig. 1). The control was used as the healthy control (without any treatment.), and the Aβ1-42 treatment group was employed as the PNU282987-treated control.

PNU282987 upregulates HSF-1, CRYAB and HSP-70 expression in astrocytes. To evaluate the effect of PNU282987 on the HSP response, astrocytes were treated with 5 µM PNU282987 at various time-points (0, 6, 12, 18 and 24 h). This resulted in the upregulation of HSF-1, CRYAB and HSP-70 (Fig. 2A and B). The upregulatory effect of PNU282987 was time-dependent and peaked at 18 h. Therefore, this time-point was selected for subsequent experiments. PNU282987-mediated upregulation of endogenous HSF-1, CRYAB and HSP-70 was signiﬁcantly antagonized by the α7 nAChR antagonist MLA (0.1 µM), indicating that α7 nAChRs was involved in the upregulatory effect of PNU282987 (Fig. 2C and D). Western blot analysis revealed that the HSF-1 knockdown (KD) effectively decreased Cryab expression at 18 h after PNU282987 treatment. However, HSP-70 expression did not exhibit a signiﬁcant change in HSF-1-KD cells compared with negative control (NC; scrambled shRNA) cells (Fig. 2E and F).

PNU282987 upregulates HSF-1, CRYAB and HSP-70 via the PI3K/Akt signaling pathway. PNU282987 upregulated p-Akt levels at various time-points (0, 5, 10, 20 and 30 min) (Fig. 3A). Moreover, pretreatment with the PI3K inhibitor LY294002 (10 µM) or the α7 nAChRs inhibitor MLA (0.1 µM) for 2 h, followed by co-incubation with PNU282987 (5 µM), indicated that MLA and LY294002 could signiﬁcantly inhibit p-Akt upregulation during PNU282987 treatment of astrocytes (Fig. 3B and C). Moreover, pre-treatment with LY294002 for 2 h signiﬁcantly inhibited the upregulation.
of HSF-1, Cryab and HSP-70 during PNU282987 treatment of astrocytes (Fig. 3D and E), indicating the involvement of the PI3K pathway in the upregulatory effect of PNU282987 (PNU282987 upregulated the expression of HSF-1, Cryab and HSP-70).

**Figure 2.** HSP reaction is activated in PNU-treated astrocytes. (A) Detection of Cryab, HSP-70 and HSF-1 expression levels in cells (treated with PNU at different time-points) using western blot analysis. (B) Statistical analysis of A. (C) Detection of Cryab, HSP-70 and HSF-1 expression in cells (treated with PNU + MLA) using western blot analysis. (D) Statistical analysis of C. (E) Detection of Cryab, HSP-70 and HSF-1 expression in cells (treated with PNU + HSF-1 shRNA) using western blot analysis. NC indicates scramble shRNA. (F) Statistical analysis of E. β-actin was used as an internal loading control. SPSS was used for statistical analysis. Data are presented as the mean ± SD; each experiment was repeated independently three times. *P<0.05 vs. the control group; †P<0.05 vs. the Pnu pre-treatment group. HSP, heat shock protein; Pnu, Pnu282987; cryab, αb-crystallin; HSP-70, heat shock protein 70; HSF-1, heat shock factor 1; MLA, methyllycaconitine; shRNA, short hairpin RNA; NC, negative control.

PNU282987 inhibits Aβ aggregation via the PI3K/Akt signaling pathway. Next, it was explored whether the PI3K/Akt pathway was involved in the inhibition of Aβ accumulation during PNU282987-treatment of astrocytes. The results revealed that PNU282987 treatment led to the inhibition of Aβ aggregation.
in astrocytes; however, pre-treatment with LY294002 for 2 h significantly attenuated this effect (Fig. 4A and B). The proposed pathway by which Pnu282987 regulates Aβ accumulation in astrocytes is presented in Fig. 4C.

Discussion

PNU282987 can inhibit Aβ aggregation in astrocytes; however, the specific mechanism underlying this effect remains to be clarified. The present results indicated that the PNU282987-mediated neuroprotection against Aβ accumulation was associated with upregulation of endogenous Cryab and HSP-70 in astrocyte cultures. Moreover, upregulation of astrocytic Cryab depended on HSF-1, and HSF-1 KD effectively decreased Cryab expression. These effects were regulated by α7 nAChRs and the PI3K/Akt signal transduction pathway in astrocyte cultures.

α7 nAChRs are ion channel receptors associated with memory and cognitive function in AD and Parkinson’s disease (PD) (27,28). These receptors are widely expressed in the human cerebral cortex, especially in astrocytes and microglia. α7 nAChRs are activated in neurons with simvastatin via the calmodulin-kinase II signaling pathway (29). Activation of α7 nAChRs has been revealed to protect neurons from the neurotoxic anticancer drug oxaliplatin (30). Hence, PNU282987, an α7 nAChR agonist, was employed to explore its neuroprotective effect in Aβ-treated astrocytes. PNU282987 enhanced astrocytes to inhibit Aβ aggregation, a process closely associated to the upregulation of HSP.

Aβ aggregates and deposits are associated with cognitive dysfunction, neuronal death and synaptic impairment in patients with AD (3,31,32). Previous studies have revealed a significant upregulation of α7 nAChR in astrocytes of AD brains that is closely associated with Aβ accumulation (14,15). Moreover, α7 nAChRs have been revealed to interact with Aβ (33). However, the relationship between α7 nAChRs and Aβ aggregation is still not clear in astrocytes. The present results indicated the involvement of α7 nAChRs and the PI3K/Akt signaling pathway in PNU282987-mediated protection against Aβ neurotoxicity, and the upregulation of endogenous Cryab
et al: Pnu282987 inhibits amyloid-β aggregation in astrocytes. Moreover, HSF-1 KD effectively decreased Cryab expression. Therefore, HSF-1 may specifically activate the expression of Cryab. Cryab can effectively prevent the accumulation and cytotoxicity of Aβs by directly combining with them; in addition HSP-70 can inhibit the production of Aβs by combining directly with APP (17,34). Subsequently, HSF-1 becomes activated in in vitro models of Pnu282987 neuroprotective activity, causing the upregulation of Cryab. Furthermore, in the present study, HSF-1 KD was associated with a notable decrease in Cryab in both control and Pnu282987-treated astrocytes but did not affect HSP-70 expression. Collectively, the present results indicated that the increase of Cryab and HSP-70 may play a key role in the Pnu282987-induced neuroprotection against Aβ aggregation in in vitro models, and the upregulation of Cryab seems to be mediated by HSF-1. The present results indicated that Cryab could directly combine with Aβs, preventing their aggregation and cytotoxicity, which is consistent with past research (17).

Previous studies have suggested an increased activation of the PI3K/Akt pathway during Tau hyperphosphorylation in AD mice (35). However, it is unclear whether the PI3K/Akt pathway is activated as a result of a neuroprotective heat shock response elicited by Pnu282987. The present results revealed an increase in Akt phosphorylation in astrocytes during Pnu282987 treatment. However, pre-treatment with the PI3K inhibitor LY294002 weakened the upregulatory effect of Pnu282987. Moreover, pre-treatment with the PI3K inhibitor for 2 h significantly inhibited the PNU282987-mediated upregulation of endogenous HSF-1, Cryab and HSP-70 in astrocytes. In addition, HSF-1 KD was associated with a marked decrease in Cryab in both the negative control and HSF-1-KD cells, which supports a role of Cryab in regulating the protective effect of HSF-1 during the treatment of cells with PNU282987.

In conclusion, in response to stress or injury induced by Aβ, astrocytes may activate heat shock response for adaptation and cell survival. The present study provided evidence that PNU282987 can significantly enhance astrocytes to inhibit Aβ accumulation by the activation of α7 nAChRs and the PI3K/Akt signaling pathway. It was further verified that upregulation of endogenous HSF-1, Cryab and HSP-70 is one of the heat shock response mechanisms for astrocyte protection.

Figure 4. PNU enhances astrocytes to inhibit Aβ aggregation via the PI3K/Akt signaling pathway. Pre-treatment with LY294002 (10 μM) for 2 h was followed by PNU (5 μM) treatment for 18 h. Finally, Aβ1-42 (1 μM) was added to the co-culture for 24 h. (A) Cell lysates were collected for immunoblot analysis of Aβ; β-actin was used as an internal loading control. (B) ImageJ was used to analyze the protein expression of Aβ, and SPSS for statistical analysis. Data are presented as the mean ± SD; the experiment was repeated independently three times. *P<0.05 vs. the Aβ group; **P<0.01 vs. the PNU + Aβ group; ***P<0.01 vs. the control group; (C) The proposed pathway by which PNU regulates Aβ accumulation in astrocytes. Aβ, amyloid-β; PNU, PNU282987; Cryab, αb-crystallin; HSP-70, heat shock protein 70; HSF-1, heat shock factor 1.
or adaption during PNU282987 treatment. Collectively, these results demonstrated the neuroprotective actions of PNU282987 in vitro, which will guide future studies exploring this mechanism in AD animal models.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the National Natural Science Foundation of China (Project no. 81360199), a Special Grant of the Central Government Supporting Local Science and Technology Development, the Science and Technology Department of Guizhou Province [Guizhou specific grant (grant no. gzwjkj2019-1-039), and the Science and Technology Fund Project of Guizhou Health and Health Commission (grant no. gzwjkj2019-1-039), and the Science and Technology Fund Project of Southwest Guizhou Autonomous Prefecture (2019-1-10).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

The first two authors contributed equally to this work. WY and ZR designed the study, ZR wrote and revised the manuscript. ZG and ZG helped perform the experiments. CA and JH performed the experiments. SL performed the analysis of the data with constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments in the present study were approved by the Animal Care Committee of Guizhou Medical University and were implemented in strict accordance with the relevant guidelines (no. 1503008).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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