Astragaloside IV ameliorates cognitive impairment and neuroinflammation in an oligomeric Aβ induced Alzheimer's disease mouse model via inhibition of microglial activation and NADPH oxidase expression

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Abstract

Microglial activation and neuroinflammation induced by amyloid β (Aβ) play pivotal roles in Alzheimer’s disease (AD) pathogenesis. Astragaloside IV (AS-IV) is one of the major active compounds of the traditional Chinese medicine *Astragalus Radix*. It has been reported that AS-IV could protect against Aβ-induced neuroinflammation and cognitive impairment, but the underlying mechanisms need to be further clarified. In this study, the therapeutic effects of AS-IV were investigated in an oligomeric Aβ (oAβ) induced AD mice model. The effects of AS-IV on microglial activation, neuronal damage and NADPH oxidase expression were further studied. Different doses of AS-IV were administered intragastrically once a day after intracerebroventricularly oAβ injection. Results of behavioral experiments including novel object recognition (NOR) test and Morris water maze (MWM) test revealed that AS-IV administration could significantly ameliorate oAβ-induced cognitive impairment in a dose dependent manner. ELISA results showed that increased levels of ROS, TNF-α, IL-1β and IL-6 in hippocampal tissues induced by oAβ injection were remarkably inhibited after AS-IV treatment. OaAβ induced microglial activation and neuronal damage was significantly suppressed in AS-IV-treated mice brain, observed in immunohistochemistry results. Furthermore, oAβ upregulated protein expression of NADPH oxidase subunits gp91phox, p47phox, p22phox and p67phox were remarkably reduced by AS-IV in western blotting assay. These results revealed that AS-IV could ameliorate oAβ-induced cognitive impairment, neuroinflammation and neuronal damage, which were possibly mediated by inhibition of microglial activation and down-regulation of NADPH oxidase protein expression. Our findings provide new insights of AS-IV for the treatment of neuroinflammation related diseases such as AD.

Keywords: Astragaloside IV; Alzheimer's disease; neuroinflammation; cognitive impairment; microglial activation; NADPH oxidase
1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. The essential role of microglia-mediated neuroinflammation in AD pathogenesis has been well demonstrated\(^1,2\). Microglia are brain innate immune cells which play pivotal roles in homeostasis maintenance and immune defenses in central nervous system (CNS)\(^3\). When suffering from brain injury or immunological stimuli such as amyloid beta (Aβ) and lipopolysaccharide (LPS), microglia become readily active and release varieties of inflammatory cytokines, including nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6)\(^4,5\). Microglial activation and attendant neuroinflammation have been demonstrated to contribute to neuronal damage and aggravate AD progression\(^6\). Therefore, inhibition of microglial activation and neuroinflammation may represent an effective strategy for the treatment of AD. In recent years, both laboratory and epidemiologic evidences have revealed that compounds with anti-inflammatory activity such as non-steroidal anti-inflammatory drugs (NSAIDs) and some natural compounds show the ability to protect neurons and cognitive function through inhibiting Aβ-induced microglial activation\(^7,10\). These findings suggest that compounds targeting microglial activation induced neuroinflammation might serve as potential approaches for AD treatment.

Astragaloside IV (AS-IV) is a triterpenoid saponin isolated from Astragalus membranaceus and A. mongholicus. AS-IV is one of the major bioactive components in traditional Chinese medicine Radix Astragali (Huangqi), which has been widely used in the treatment of immune disorders, cardiovascular and hepatic diseases for a long history\(^11,12\). Pharmaceutical studies have shown the prominent anti-inflammatory effect of AS-IV, which was found to possess anti-inflammatory activity via inhibiting NF-κB activation and inflammatory gene expression, as well as suppression of autophagy\(^13-15\). Recent in vivo studies have revealed the protective effects of AS-IV in murine AD models\(^16,17\). However, it is unknown that whether AS-IV can ameliorate oligomeric Aβ (oAβ) induced neuroinflammation and cognitive dysfunction. The present work aimed to investigate the in vivo therapeutic effect of AS-IV against oAβ-induced neuroinflammation and memory deficit in an AD mouse model. To discover the underlying mechanism, the effects of AS-IV on microglial activation and protein expression of NADPH oxidase subunits were further investigated.

2. Materials and Methods

2.1. Preparation and identification of oAβ peptides

Aβ1-42 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). OAp peptides were prepared according to the method previously described\(^18\). Briefly, Aβ1-42 was dissolved in 1 mM hexafluoroisopropanol, aliquoted and dried with nitrogen gas and further dried under vacuum. The residual peptide was dissolved in DMSO and diluted with ultrapure water, then 2 M Tris-base solution (pH 7.6) was added to a final concentration of 1 mg/ml, and shaken for 30 min at room temperature on a shaker. Prepared OAp was identified by Tricine-SDS-polyacrylamide gel electrophoresis\(^19\). The OAp solution was sampled and electrophoresed for 3 h with initial current at 15 mA and then at 25 mA. After electrophoresis, the gel was fixed and stained with Coomassie brilliant blue, then decolorized until the band was clear.

2.2. Regents

AS-IV was purchased from Xi’an Linhe Biotechnology Co., Ltd. (Xi’an, China, purity > 98%, detected by HPLC). Enzyme linked immunosorbent assay (ELISA) kits including ROS, TNF-α,
IL-1β and IL-6 were obtained from BOSTER Biological Technology Co. Ltd. (Wuhan, China). Mouse primary antibodies including gp91phox, p22phox, p47phox, p67phox and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Animal experiment procedure
Male ICR mice weighing about 22-25 g were obtained from Experimental Animal Center of Ningxia Medical University (Yinchuan, China). All animal procedures were performed in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and approved by Ethic Committee of Ningxia Medical University (NXMU2014-124). Mice were housed under constant environmental conditions at 25°C with food and water freely available, with a 12 h/12 h dark/light cycle. Sixty mice were divided into six different groups (10 mice per group) randomly: control group (sham-operation with intragastric administration of saline), model group (oAβ injection with intragastric administration of saline), donepezil group (oAβ injection with intragastric administration of donepezil 5 mg/kg/d) and high, middle, low dose AS-IV groups (oAβ injection with intragastric administration of AS-IV 80, 40 and 20 mg/kg/d respectively). Mice were anesthetized with an intra-peritoneal injection of 350 mg/kg chloral hydrate and intracerebroventricularly (i.c.v.) injected with 2 nmol oAβ (4 μL) according to the stereotaxic atlas of mouse brain to establish AD mice model.

While the control group mice were i.c.v. injected with the same volume of saline. The intragastric administration lasted for 21 days and the behavioral tests were performed 15~21 days after oAβ injection. Then the mice were sacrificed by cervical dislocation in day 22. Iba-1 immunohistochemistry were performed to evaluate microglial activation. Levels of ROS, TNF-α, IL-1β and IL-6, as well as expressions of NADPH oxidase subunits gp91phox, p47phox, p22phox, and p67phox in hippocampal tissue were assessed by ELISA and western blotting assay.

2.5. Novel object recognition test
NOR tests were performed as described in a test box (50 cm × 50 cm × 30 cm), 15~17 days after oAβ injection. Firstly in the habituation period, all mice were allowed to explore freely in the box for 3 min to habituate to the environment without objects. Secondly in the training session, two identical objects were placed into the test box and each mouse was allowed to explore in the box freely for 3 min. The time mouse spent in exploring each object was recorded. In the test session started 24 h later, one of the original object was replaced by a novel object (similar in size but different in shape and color) and each mouse was allowed to explore for 3 min. The time mouse spent in exploring the novel and the original objects was recorded respectively. The mice were considered to be exploring when they were touching or sniffing the object. Preference index (PI, %) were calculated as the percentage of novel object exploring time in total exploring time.

2.6. Morris water maze test
Morris water maze (MWM) test was performed 18~21 days after oAβ injection as described to evaluate the effects of AS-IV on oAβ induced spatial learning and memory impairment. Briefly, the test was carried out in a 1.0 m diameter tank which was divided into four quadrants. A 6.5 cm diameter hidden transparent platform was fixed 0.5 cm below the water surface in the center of the IV quadrant. Firstly, the place navigation test was carried out. Mice were released into water from the edge of each quadrant at the water-level individually and allowed to swim freely for 90 s until they found the hidden platform. If a mouse failed to find the platform within 90 s, it was transferred onto the platform and stayed for 30 s. Training lasted for 3 consecutive days and mice
were trained two times each day. The place navigation test was performed 24 h after the last training and the time taken to find the platform was recorded as escape latency. After that, the spatial probe test was performed, in which the platform was removed and mice were individually released into pool from the edge of II quadrant. Each animal was allowed to swim 90 s freely to search the platform. The ratio of time spent in the former platform quadrant (IV) and number of times of crossing the former platform were recorded. All data were analyzed by the WMT-100S analysis system (Techman Software, Chengdu, China).

2.7. Thioflavin-T staining

Thioflavin-T (ThT) staining was used to quantitatively detect the Aβ deposition in the mice brain 3 weeks after intracerebroventricular injection of oAβ\(^{19}\). The fixed mouse brain tissue samples were rinsed with water, dehydrated and embedded with paraffin, cut into 3-5 μm thick sections with a slice machine. The sections were flushed with 0.1% aqueous ThT solution (AAT Bioquest, California, USA), dyed at room temperature for 20 minutes, washed 3 times for 5 minutes each, and observed with fluorescence microscope after sealing. The Aβ deposition area was quantified by ImageJ software (version 1.44, National Institutes of Health, Bethesda, MD, USA).

2.8. Determination of ROS, TNF-α, IL-1β and IL-6

Hippocampal tissues of sacrificed mice were homogenized in ice-cold homogenization buffer. Homogenates were then centrifuged (10000 g, 4˚C) for 10 min. Different ELISA kits were used to measure the levels of ROS, TNF-α, IL-1β and IL-6 in the supernatant according to the manufacturer’s protocol.

2.9. Western blotting assay

Protein expression of NADPH oxidase subunits including gp91phox, p47phox, p22phox, and p67phox in mice hippocampus were measured by western blotting. The mice hippocampal tissues were lysed in ice-cold triple-detergent lysis buffer and centrifuged at 10000×g for 10 min. The protein concentrations in supernatant were measured using BCA Protein Assay Kit (Pierce /Thermo Scientific, Rockfold, IL, USA). Equal amounts of protein (60 μg) were subjected to 12% SDS-PAGE (Ameresco, Solon, OH, USA) and the proteins were then transferred to 0.45 μm PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk in Tris-buffered saline-Tween (TBST) and incubated overnight with primary antibodies (all 1:500 dilution) at 4˚C. After thorough washing with PBST, membranes were incubated with horseradish peroxidase conjugated secondary antibodies (1:4000 dilution, Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. The membranes were then washed three times with TBST and immunoreactive bands were visualized by incubating the membranes with the enhanced chemiluminescence reagent (ECL, Beyotime Biotechnology, Shanghai, China). β-actin was used as an internal control for standardization. Relative intensities of bands were quantified with ImageJ software (version 1.44, National Institutes of Health, Bethesda, MD, USA).

2.10. Immunohistochemistry

Immunoreactive expression of Iba-1 and NeuN in mice brain was detected by immunohistochemical staining. Mice brains were embedded in paraffin and 5 μm thick sections were prepared for immunohistochemical staining. Sections were treated with 1% hydrogen peroxide in PBS for 15 min to quench endogenous peroxidase activity. The sections were incubated separately with a primary antibody (Iba-1, 1:800 dilution, Wako, Osaka, Japan; NeuN, 1:500 dilution, Chemicon, Temecula, CA, USA) at 4˚C overnight. Then, they were incubate with
biotinylated anti-rabbit secondary antibody (1:200 dilution, Vector, Burlingame, CA, USA) for 90 min, followed by incubation in avidin-biotin complex (1:100 dilution) for 1 h at room temperature. The sections were incubated with DAB in 0.05 M tris-buffered saline (pH 7.6) to visualize peroxidase activity. The images were captured by an a digital camera attached to a optical microscope (BX51, Olympus, Tokyo, Japan) to quantify the immunoreactivity of Iba-1 and NeuN. The immunoreactive area fractions of Iba-1 and NeuN were analyzed using ImageJ software and manual threshold adjustment was performed in selection of positive area. Data were expressed as percentages compared to the control group.

2.11. Data Analysis
Results were expressed as the mean ± S.D.. Student’s t-test and one-way ANOVA were used for statistical analysis (SPSS 16.0; SPSS, Chicago, IL, USA). The p values less than 0.05 were considered statistically significant for all analyses.

3. Results

3.1. Formation of oligomerized Aβ
The aggregation of Aβ was determined by polyacrylamide gel electrophoresis. A clear band between 10-15 KD which may represent the formation of Aβ dimer was observed after 20 min incubation at room temperature. During incubation of 30 min, the color of the band deepened, indicating Aβ aggregation increased significantly (Fig. 1).

3.2. AS-IV ameliorates oAβ induced cognition impairment
Behavioral studies were performed to evaluate the effect of AS-IV on cognitive impairment in oAβ-induced AD mice model. NOR test was employed to investigate the protective effect of AS-IV on novel-object recognition ability. Comparing with the control group, preference index of the model group mice injected with oAβ remarkably decreased. While AS-IV or donepezil treatment groups showed a remarkable recovery in preference index (Fig. 2A). MWM test was performed to investigate the protective effect of AS-IV on spatial learning and memory function. In the place navigation test, oAβ injected mice showed significantly longer escape latency compared with those in the control group. After administration of AS-IV or donepezil, the escape latency was remarkably decreased (Fig. 2B). In the spatial probe test, both time spent in the target quadrant and times of passing through the platform were decreased in oAβ-injected mice, which were remarkably reversed by AS-IV or donepezil treatment (Fig. 2C and 2D). The above results reveal that AS-IV treatment can ameliorate oAβ induced cognitive function impairment in AD mouse model.

3.3. ThT staining confirmed the deposition of oAβ
ThT staining was used to detect the existence of Aβ deposition in mice brain. Results showed that there was obvious green fluorescence in the cortex and hippocampus of the model group mice (Fig. 3). The results revealed that 3 weeks after intracerebroventricular injection of oAβ, the Aβ deposition was found in the brain tissue of mice, and the Aβ deposition area in the model group was significantly higher than that in the control group.

3.4. AS-IV inhibits oAβ-induced inflammatory mediators production
The effects of AS-IV on pro-inflammatory mediators release in mice hippocampus were evaluated.
It is found that oAβ injection could significantly increase the levels of ROS, TNF-α, IL-1β and IL-6 comparing with control group. While AS-IV treatment could remarkably inhibit production of inflammatory mediators in mice hippocampus (Fig. 4). The results show that AS-IV could potently inhibit oAβ induced neuroinflammation.

3.5. AS-IV inhibits oAβ-induced microglial activation and neuronal damage

Iba-1 immunohistochemical staining was used to evaluate the effects of AS-IV on microglial activation in oAβ-injected mice brain. Comparing with control group, the Iba-1 positive area fraction remarkably increased in brains of oAβ-injected mice. While the Iba-1 positive areas were remarkably decreased after AS-IV treatment (Fig. 5), suggesting that AS-IV could potently inhibit oAβ-induced microglial activation in vivo. NeuN immunohistochemical staining was used to observe the effect of AS-IV on neuronal injury in the brain of mice. Comparing with control group, the number of neurons in the model group decreased significantly and the intercellular space increased, result in decrease of NeuN positive area. After AS-IV treatment, the number of neurons increased and cells were arranged tightly, showing a remarkable increase of NeuN positive area (Fig. 6), which suggest that AS-IV show potent protective effect against oAβ-induced neuronal damage.

3.6. AS-IV down-regulate oAβ-induced NADPH oxidase subunits protein expression

NADPH oxidase has been proved to play a pivotal role in microglia mediated neuroinflammation. NADPH oxidase subunits including catalytic subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox, and p40phox) are required for enzyme activation. Western blotting results showed that oAβ injection could increase the protein expressions of gp91phox, p22phox, p47phox and p67phox in AD mice brain, which were down-regulated by AS-IV treatment (Fig. 7). The results suggested that the inhibitory effects of AS-IV on oAβ-induced microglia activation and neuroinflammation were possibly mediated through regulation of the protein expressions of NADPH oxidase subunits.

4. Discussion

In this study, it was demonstrated that AS-IV could ameliorate learning and memory impairment, reduce the release of inflammatory cytokines, and inhibit microglia activation in oAβ-induced AD mice. Furthermore, western blotting results indicated that AS-IV administration could down-regulate protein expressions of NADPH oxidase subunits gp91phox, p47phox, p22phox and p67phox. Our results revealed that AS-IV ameliorated oAβ-induced cognitive deficits and neuroinflammation via down-regulating NADPH oxidase expression, which have not been reported yet. This finding provide a new insight for AS-IV in the treatment of neuroinflammatory-related diseases such as AD. Consistent with the previous research, donepezil was found to exert potent effects on inhibition of neuroinflammation and microglial activation.

Lines of evidence have revealed that AS-IV is a bioactive compound with potent anti-inflammatory properties, but the effect of AS-IV on neuroinflammation and related mechanism is not well elucidated. In LPS induced BV2 and primary microglia cells, AS-IV could significantly reduce the release of inflammatory mediators through activating NRF2/HO-1 via ERK signaling pathway. In a report investigating the effect of AS-IV on the microglia activation
stimulated by LPS, AS-IV was found to exert anti-inflammatory effect through inhibiting TLR4/NF-κB pathway and converting microglia from inflammatory M1 phenotype to anti-inflammatory M2 phenotype. Moreover, it was reported that AS-IV inhibits microglial activation partially by activating glucocorticoid receptor mediated signaling pathway. Results of this study clearly showed that AS-IV treatment could significantly ameliorate αAβ induced microglial activation and neuroinflammation in mice brain, providing more evidence for the anti-neuroinflammatory effect of AS-IV in central nervous system.

Nerve inflammation and oxidative damage induced by NADPH oxidase derivatives are the key to accelerate and aggravate AD. Microglia cells have been proved to be the primary resources of NADPH oxidase in central nervous system. Activated microglia produce various neurotoxic inflammatory factors, among which ROS has an pivotal role in microglia-mediated neuroinflammation. NADPH oxidase-derived ROS not only participate in host defense via removing pathogens and inducing a variety of antioxidant enzymes in host cells, but also act as secondary messenger to enhance gene and protein expression of various pro-inflammatory factors. Previous study using human umbilical vein endothelial cells showed that AS-IV could down-regulate protein expression of Nox4, a homologue of gp91phox and the most abundant catalytic subunit of NADPH oxidase in umbilical vein endothelial cells. In the present study, it was found that AS-IV could remarkably reduce ROS production in αAβ-injected mice brain. Although AS-IV reportedly enhances the antioxidant capacity and scavenges ROS by increasing the levels of antioxidant enzymes such as SOD, GSH-Px, and CAT, a new finding from this study was that AS-IV could potently down-regulate αAβ-induced protein expression of microglial NADPH oxidase subunits and consequent ROS production. Considering previous study which found that the decreased expression of p47 subunit can decrease the activity and expression of NADPH oxidase, AS-IV may inhibit the expression of NADPH oxidase in αAβ-injected mice by reducing the expression of NADPH oxidase p47 subunit. Whether AS-IV could affect the translocation and phosphorylation of NADPH oxidase subunits and thus affecting activity of NADPH oxidase need to be further investigated.

AS-IV also showed remarkable inhibitory effect on production of TNF-α, IL-1β and IL-6 in the αAβ-injected mice brain. These pro-inflammatory cytokines are regarded as remarkable substances in microglia activation. Our previous study showed that stand-alone αAβ induced release of the three inflammatory factors in cultured microglia cells, and NADPH oxidase play an key role in this effect. Therefore, AS-IV induced down-regulation of NADPH oxidase expression could reduce the release of pro-inflammatory factors from microglia activated by αAβ. In recent years, it is generally believed that the increase of cytokines and chemokines released by activated microglia leads to chronic neuroinflammation, which is a pivotal part of the cause of neuron damage and neurodegeneration in the brain of AD. Thus, AS-IV attenuates αAβ-induced inflammatory cytokines release via inhibiting microglia activation and NADPH oxidase expression, which contributed to the protective effect of AS-IV on learning and memory function in AD mice.

Taken together, this study reported that astragaloside IV could effectively ameliorate cognitive impairment in αAβ induced AD mice model. Further investigation showed that AS-IV could inhibit αAβ induced microglial activation and subsequent neuroinflammation. Moreover, NADPH oxidase subunits including gp91phox, p47phox, p22phox and p67phox might play pivotal roles in the anti-AD effects of AS-IV. The results suggest that AS-IV is a valuable natural product with
therapeutic potential for the treatment of AD.

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Conflict of interest
The authors declare no conflict of interest.
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Fig. 1. Aggregation of oAβ on Tricine-SDS-PAGE gel. Aβ 1-42 (1 mg/ml) was incubated in a mixture of 100% DMSO and double distilled aqueous solution for 30 min, sampled every 10 min, and the sample was run on 10% polyacrylamide gel. The molecular weight standard is marked on the left.
Fig. 2. Effects of AS-IV on cognitive function in oAβ-induced AD mice. OAβ-injected AD mice model were administrated by gavage with saline, AS-IV (20, 40 and 80 mg/kg) or donepezil (5 mg/kg). (A) Preference index (PI, %) in NOR test. (B) Escape latency in place navigation test. (C) The percentage of time spent in target quadrant and (D) number of platform crossing times during spatial probe test. Each bar represents the mean ± S.D. of data (n = 10). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with control group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with oAβ group.
Fig. 3. Deposition of oAβ in brain tissue of mice 3 weeks after injection of oAβ oligomer. ThT staining showed that oAβ oligomers were deposited in hippocampal (A) and cortical (B) in brain tissue (Scale bar = 200 μm). The ThT positive area was measured by ImageJ software. Each bar represents the mean ± S.D. of data from three independent experiments (n = 3). **P < 0.01 and ***P < 0.001 compared with the control group. (Color figure can be accessed in the online version.)
Fig. 4. Effects of AS-IV on pro-inflammatory factors release in hippocampus of oAβ-induced AD mice. Oαβ-injected AD mice were administrated by gavage with saline, AS-IV (20, 40 and 80 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the brain hippocampal tissues of mice were collected for ROS (A), TNF-α (B), IL-1β (C) or IL-6 (D) concentration measurement. Each bar represents the mean ± S.D. of data from three independent experiments (n = 3). ###P < 0.001 compared with control group. *P < 0.05 and **P < 0.01 compared with oAβ group.
Fig. 5. Effects of AS-IV on microglial activation in oAβ-induced mice brain. OAβ-injected AD mice model were administrated by gavage with saline, AS-IV (20, 40 and 80 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the mice brain tissues were collected and activation of microglia was visualized by Iba-1 staining (A: a. Con, b. oAβ, c-e. AS-IV 20, 40 and 80 mg/kg, f. donepezil. Scale bar = 100 μm). Quantification analysis of the Iba-1-stained cells (b) was conducted by measuring the fractions of Iba-1-immunoreactive areas. Values are expressed as mean ± S.D.. ***P < 0.001 compared with control group. **P < 0.01 and ***P < 0.001 compared with oAβ group. (Color figure can be accessed in the online version.)
**Fig. 6.** Effects of AS-IV on neuronal damage in oAβ-injected mice brain. AD mice were administrated by gavage with saline, AS-IV (20, 40 and 80 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the mice brain tissues were collected and the damage of neurons was visualized by NeuN staining (A: a. Con, b. oAβ, c-e. AS-IV 20, 40 and 80 mg/kg, f. donepezil. Scale bar = 100 μm). Quantification analysis of the NeuN-stained cells (b) was conducted by measuring the fractions of NeuN-immunoreactive areas. Values are expressed as mean ±S.D.. ###P < 0.001 compared with control group. *P < 0.05 and **P < 0.01 compared with oAβ group. (Color figure can be accessed in the online version.)
Fig. 7. Effects of AS-IV on protein expression of NADPH oxidase subunits gp91phox, p47phox, p22phox and p67phox in hippocampal tissues of oAβ induced AD mice. oAβ-injected AD mice were administrated by gavage with saline, AS-IV (20, 40 and 80 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the mouse brain hippocampal tissues were collected and protein expression of NADPH oxidase subunits were evaluated by western blotting assay. β-actin protein was used for standardization, and ImageJ software (version 1.44) was used for band pattern analysis. Values are expressed as mean ± S.D. **P < 0.01 and ***P < 0.001 compared with control group. *P < 0.05 and ***P < 0.001 compared with oAβ group.