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Soluble oligomers and fibrillar species of amyloid β-peptide differentially affect cognitive functions and hippocampal inflammatory response

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ABSTRACT

Two major active species of β-amyloid protein (Aβ), fibrillar Aβ1-42 (FAβ) and soluble Aβ1-42 oligomers (AβO), are known to play important roles in the pathogenesis of Alzheimer's disease. However, the differences between them are largely unknown. In this study, we explored the effects of FAβ and AβO on cognitive functions and hippocampal inflammatory response through a 30-days infusion of FAβ or AβO (144 pmol/d) into the left lateral ventricles of the rat brain. Morris water maze showed that the impairment of learning and memory functions was much more significant in the AβO-infused rats, compared to the FAβ-infused rats. AβO-induced neurodegeneration and ultrastructure damage in CA1 neurons were more remarkable than those induced by FAβ. Compared to FAβ, AβO exerted more potent effects on the expressions of inflammatory factors toll-like receptor 4 and TNF-α and activation of NF-κB signaling. Taken together, our results from *in vivo* model demonstrate that AβO is more neurotoxic than FAβ, and this neurotoxicity may be related to NF-κB-medicated inflammatory response.

1. Introduction

Alzheimer's disease (AD) is the most common age-related disorder which is characterized by memory loss and cognitive deficit. Accumulation of amyloid-β peptide (Aβ) in brain is one key pathological event of AD [1]. According to the classic amyloid cascade hypothesis, Aβ is derived from amyloid precursor protein, which is processed by β and γ secretases into peptides predominantly 40 (Aβ1-40) and 42 amino acids (Aβ1-42) in length. Aβ1-40 and Aβ1-42 are deposited into amyloid plaques to form one of the pathologic hallmarks of AD. So far, Aβ1-42 has been known as the more toxic form of Aβ, and the increased ratio of Aβ1-42 to Aβ1-40 is associated with familial forms of AD [2].

More recent findings indicate that Aβ dimers, small soluble Aβ oligomers and membrane-bound Aβ oligomers, are highly toxic and are associated with memory dysfunction in the early stage of AD [3]. The major water-soluble oligomers in most AD cases have been identified as Aβ1-42. Aβ1-42 aggregates from Aβ1-42 oligomers (AβO) and fibrillar Aβ1-42 (FAβ) play dominant roles in AD pathogenesis according to the classic amyloid cascade hypothesis. Furthermore, *in vitro* studies have suggested more toxicity in AβO than FAβ. AβO exerted cytotoxic effect 10-fold more than FAβ in cultured Neuro-2A neuroblastoma cells [4]. Freshly dissolved AβO bound to the lipid bilayer of biomimetic membranes with 2-fold more avidity than FAβ, and caused 2-fold more disruption of ganglioside-containing lipid membrane [5]. To date, however, earlier suggestion about the difference between AβO and FAβ has remained largely unsupported by direct and firm experimental evidence. In this study, we infused FAβ or AβO into the left lateral ventricles of the rat brain, and investigated their effects on learning and memory functions. Previous studies have disclosed that chronic inflammation in central nerve system participated in Aβ-induced neurotoxicity, possibly involving transcriptional signaling, such as NF-κB [6]. Therefore, we also investigated inflammatory response and NF-κB activation in hippocampus, the major region related to learning and memory functions.

2. Materials and methods

2.1. Reagents

Aβ1-42 (A9810), dimethyl sulfoxide, HEPES and mouse anti-β-actin antibody (A1978) were purchased from sigma. High-density lipoprotein (HDL) was purchased from Millipore. Rabbit anti-Toll-like receptors 4 (TLR4, 2219S), anti-NF-κB p65 antibody (4764s),

Abbreviations: Aβ, β-amyloid protein; FAβ, fibrillar Aβ1-42; AβO, soluble Aβ1-42 oligomers; AD, Alzheimer's disease; HDL, high-density lipoprotein; MWM, morris water maze.

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and anti-IκBα antibody (9242s) were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated 2nd antibodies were purchased from Zhongshan Jinqiao biotechnology (Beijing, China). TNF-α enzyme linked immunosorbent assay (ELISA) kit (RTA00) was purchased from R&D Systems. Real time PCR kit (A6001) was purchased from Promega. Ham's F-12 culture media (phenol red-free) was purchased from BioSource (Camarillo, CA).

2.2. Preparations of FAβ and AβO

Preparations of FAβ and AβO were performed according to previous reports [4,7,8]. Aβ1-42 peptide was first dissolved to 1 mM in hexafluoroisopropanol in sterile microcentrifuge tubes. The hexafluoroisopropanol was removed under vacuum in a Speed Vac. The peptide was then resuspended in dimethyl sulfoxide to 5 mM. For preparation of AβO, Ham's F-12 was added to bring the peptide to 100 μM, and then incubated at 4 °C for 24 h. The 100 μM of AβO was then diluted to final concentration of 20 μM in HEPES buffer (PH 8.0) containing 0.16% HEPES and 0.025% HDL. For preparation of FAβ, 10 mM HCl was added to bring the peptide to final concentration of 20 μM, and incubated for 24 h at 37 °C. The vehicles which dissolved FAβ (vehicle 1) or AβO (vehicle 2) were used as controls. Prepared AβO was examined under electron microscope (80,000×). AβO appeared as small globular structures with diameter 12–25 nm. No thread-like substance was observed (Suppl Fig. 1).

2.3. Animals and surgery procedure

Adult male SD rats (200–300 g) were housed singly with a 12 h light–dark cycle (lights on at 7 a.m.). Animal experiments were conducted in accordance with approved institutional animal care procedures. FAβ or AβO was infused into the left lateral ventricles according to previous descriptions [9,10]. The rats were anesthetized with 4% chloral hydrate intraperitoneally and positioned in a stereotaxic apparatus. Under aseptic conditions, a stainless-steel cannula was inserted into the left lateral ventricles (2.0 mm left and 1.2 mm posterior to bregma, and 4.0 mm below skull surface). A miniosmotic pump was connected to the cannula, and placed in the subcutaneous sac of neck back. *The pump velocity was 0.3 μl/h. The total 4320 pmol of AβO or FAβ was infused according to previous report* [11].

The neurologic functions of the rats were evaluated based on the criteria of Longa EZ five-point score [12]. The rats with score 2 or more were used in further experiments. We also confirmed the success of surgery after Morris water maze (MWM). The rats were anesthetized, and the lower and upper jaws were removed to expose cranial bases. Blue ink was injected through cannula. That blue ink came out from cranial bases indicated successful surgery.

2.4. MWM

To evaluate the effects of FAβ and AβO on spatial learning and memory functions, we performed MWM at the 31st day after chronic infusion. Place navigation test was assessed four trials a day for a total of four consecutive days. A hidden platform (10 cm wide) remained in a fixed location throughout testing. The rats were placed in the maze facing the pool wall from east, north, west or south location, and were allowed to swim for 180 s until they found the platform. Mean escape latency (the time taken to find the platform) of four trials per day was calculated. Probe trial was performed after place navigation test, whereby the platform was removed. The frequency rats crossed the previous platform position, and the ratio of time and distance spent in the previous platform quadrant in 120-s period were analyzed as the indicators to evaluate memory function.

2.5. Tissue preparation for electron microscopy

The hippocampal sections (70 μm) were fixed in 1% osmium tetroxide for 2 h, dehydrated in a graded ethanol series (30–50–70–95–100%), and embedded in the mixture of acetone and epoxy resin at 60 °C for 48 h. Small pieces of samples (approximate 1 mm^3 in size) containing hippocampal CA1 region were cut out of embedded sections, and cut into ultra-thin sections (1 μm) with ultra microtome. The ultra-thin sections were stained with 2% uranyl acetate for 30 min and 1% lead citrate for 15 min. Images were captured with H-7500 transmission electron microscope. Some ultra-thin sections were HE stained, and degenerated cells were observed under light microscope (400×).

2.6. Real time RT-PCR

Real-time RT-PCR was applied to evaluate mRNA expressions of TLR4 and TNF-α according to previous report [13]. Total RNA from the hippocampus was extracted, and reversely transcribed into cDNA. The resultant cDNA was amplified by real-time PCR with designed primers. TLR-4 primers were sense: 5′TGAGAAACGAGA TGGTAAAGAATT3′ and antisense: 5GTGGAAGCCTTCCTGGAT GATG3′. TNF-α primers were sense: 5′CGTCGTAGCAAACCA CCAAGC3′ and antisense: 5′ATGGCAGAGAGGAGGCTGACT3′. β-actin primers were sense: 5′GACAGGATGCAGAAGGAGATTACT3′ and antisense: 5′TGATCCACATCTGCTGGAAGGT3′. Melting curve, which was measured immediately after amplification, showed single product peak, indicating good product specificity.

2.7. Western blot

The protein levels of TLR4, IκBα and NF-κB p65 from the hippocampus were assessed with Western blot. Whole tissue proteins were separated electrophoretically in 4–12% SDS–PAGE gels, and transferred to nitrocellulose membranes. After a 1 h block with 2.5% nonfat milk, the membranes were incubated with anti-TLR-4 antibody (1: 1000), anti-IκBα antibody (1:1000), anti-NF-κB p65 antibody (1: 1000) or anti-β-actin antibody (1:4000) at 4 °C overnight, and were followed by a 1 h incubation with horseradish peroxidase-conjugated 2nd antibody (1:2000). The membranes were adequately washed with tris-buffered saline containing Tween20 after each treatment with antibody. The membranes were developed with enhanced chemiluminescence reagent and then exposed to X-ray film. The protein levels of TLR4, IκBα and p65 were expressed as the ratio of band optical intensity to β-actin.

2.8. ELISA

TNF-α protein level was assessed with ELISA. The homogenates from the hippocampus were centrifuged at 10,000 g for 10 min at 4 °C. The concentration of TNF-α in supernatant was measured with ELISA according to the protocol recommended by the manufacturer.

2.9. Statistical analysis

Data were presented as mean ± SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA), and followed by Dunnett's test for multiple comparisons. $p < 0.05$ was considered statistically different.

3. Results

3.1. Learning and memory functions

To compare the effects of FAβ and AβO on spatial learning and memory functions, we performed MWM at the 31st day after continuous infusion of FAβ or AβO into the left lateral ventricles. We measured the escape latency in place navigation test at consecutive 4 days to decide learning ability (Fig. 1A). Our results showed that the mean escape latency in all groups decreased in a day-dependent manner. No significant difference was observed between vehicle 1 and vehicle 2 groups. Infusion of AβO and FAβ significantly increased the mean escape latency. Longer escape latency appeared in the AβO group, compared to the FAβ group.

After escape latency test, we performed probe trial to determine memory function. The rats that learn the platform position are expected to swim greater distance and longer time in the platform quadrant [14]. No significant difference was observed between vehicle 1 and vehicle 2. Both FAβ and AβO infusion significantly reduced the frequency of crossing the platform position, and reduced the time and distance spent in target quadrant. The extent of reduction was more remarkable in the AβO group than that in the FAβ group (Fig. 1B–D).

3.2. Neurodegenerative changes

To investigate whether the impairment of learning and memory functions was linked to neurodegeneration, we examined histopathology and ultrastructure of the hippocampal CA1 neurons. HE staining showed that membrane shrinkage, nucleus pyknotic and intensive blue-stain in cell body were observed in the degenerated cells in the hippocampal CA1 region, as arrows indicated (Fig. 2A). There were few degenerated cells in the vehicle 1 and vehicle 2 groups. The number of degenerated cells increased significantly in the FAβ and AβO groups. The increase was more in the AβO group than that in the FAβ group. Disorder of neurons array was also observed in the AβO group (Fig. 2A).

We examined the ultrastructure of the hippocampal CA1 neurons using electron microscopy (Fig. 2B). The results showed that there was no abnormal ultrastructure change in the vehicle 1 and vehicle 2 groups. Chromosomes were well-distributed; nuclear membrane was clear; and mitochondria and ribosomes were abundant. In the FAβ and AβO-infused group, especially in the AβO-infused group, there were markedly abnormal ultrastructure changes in the hippocampal CA1 neurons: the quantity of organelles decreased significantly; chromosomes aggregated edgily; endoplasmic reticulum expanded; and *mitochondria network fragmentation* and ridge disrupted, disappeared, or vacuolized.

3.3. Expressions of inflammatory factors

Inflammation is one key hallmark of AD. Amyloid deposition is associated with activation of surrounding microglia, which produces robust inflammatory response [15]. Aβ can trigger microglial activation by interacting with several TLRs, including TLR4 [6]. Thus, we compared the effects of FAβ and AβO on the expressions of TLR4, as well as TNF-α in mRNA (Fig. 3A) and protein levels (Fig. 3B). Our results did not reveal significant difference between the vehicle 1 and vehicle 2 groups. Significant increase of expression of TLR-4 and TNF-α in mRNA and protein levels appeared in the FAβ and AβO groups. AβO induced higher expressions of TLR4 and TNF-α than FAβ in both mRNA and protein levels.

3.4. Activation of nuclear transcription factor NF-κB

Transcription factor NF-κB is activated by FAβ and AβO in cultured microglia and astrocytes [6,16]. We here compared the difference of NF-κB activation in the hippocampus between FAβ and AβO treatment. We examined the protein levels of IκBα and NF-κB p65 using western blot (Fig. 4). IκBα, NF-κB p50 and p65 form an inactive complex in intact condition. Once stimulated,

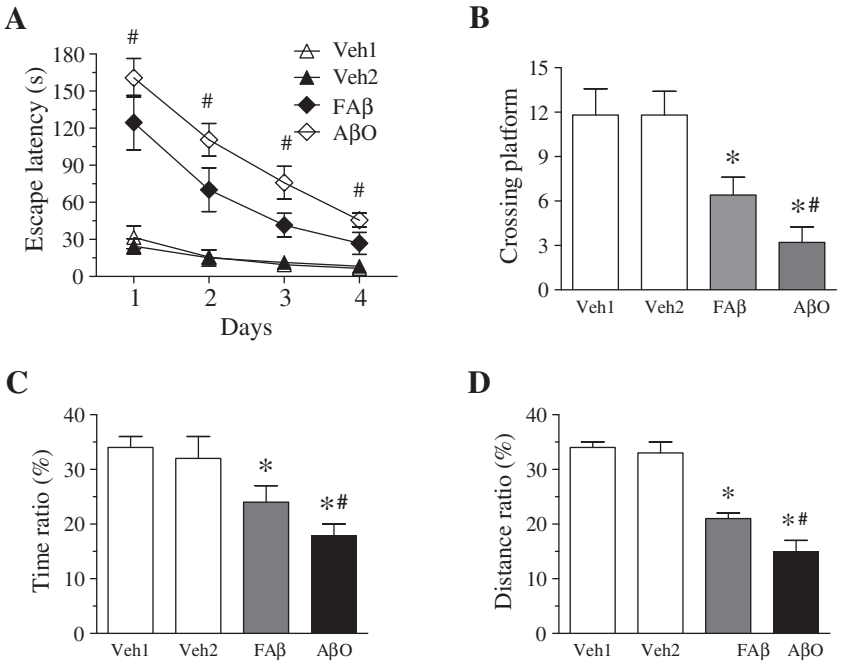

Fig. 1. Spatial learning and memory impairment induced by FAβ and AβO. MWM was performed to evaluate spatial learning and memory after 31 days of infusion of FAβ or AβO. The mean escape latency during place navigation test (A), the frequency of crossing platform position (B), the ratio of time (C) and the distance (D) spent in the target quadrant during probe trials were shown. Results were presented as mean ± SEM ($n = 15$). $^{*}p < 0.05$ vs. the vehicle 1 (Veh1) or vehicle 2 (Veh2) group. $^{\#}p < 0.05$ vs. the FAβ group.

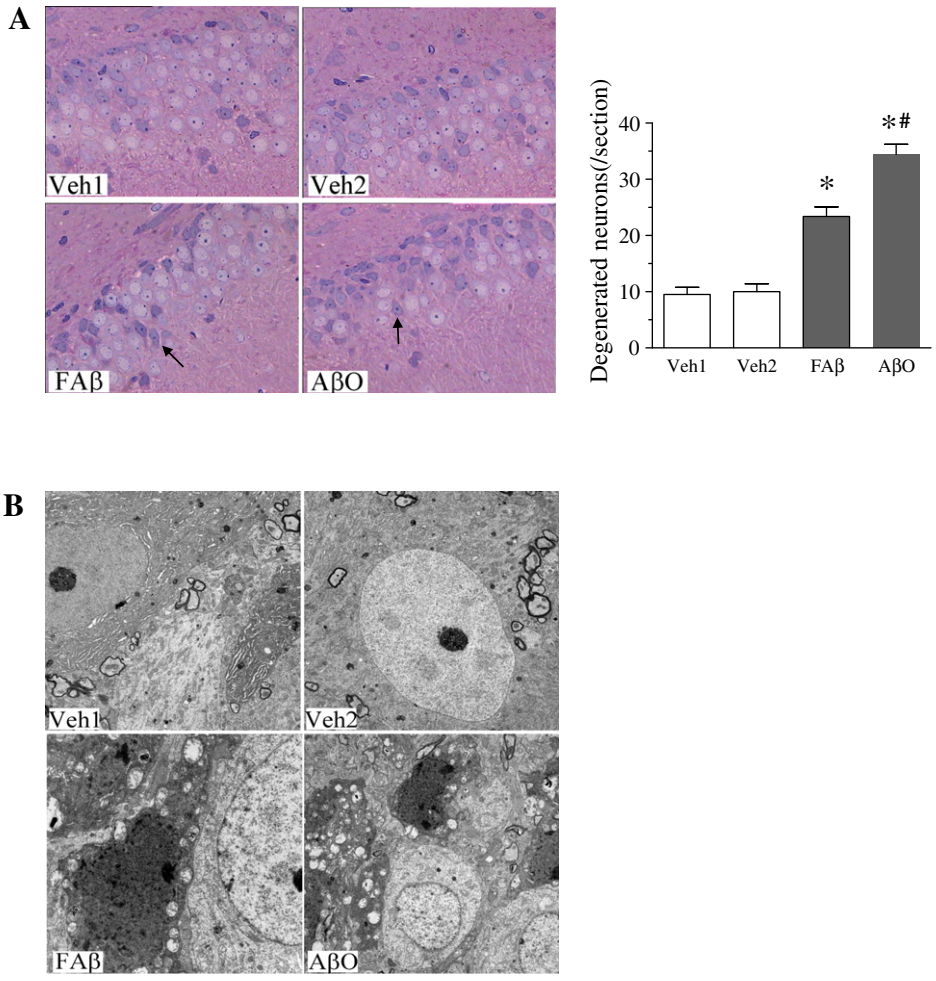

Fig. 2. Neurodegeneration and ultrastructure changes induced by FAβ and AβO. The rats were sacrificed after MWM, and the hippocampal sections were used to examine cells degeneration with HE staining (A) and ultrastructure impairment (B) under light microscope (400×) and electron microscope (10,000×), respectively. The representative sections from 6 rats in each group were shown. The black arrows indicated the degenerated neurons. $^{*}p < 0.05$ vs. the vehicle 1 (Veh1) or vehicle 2 (Veh2) group. $^{\#}p < 0.05$ vs. the FAβ group.

IκBα is phosphorylated and degraded, which consequently releases active NF-κB p65 and p50 to exert transcription function. The levels of IκBα and p65 were similar between the vehicle 1 and vehicle 2 groups. Both FAβ and AβO significantly decreased IκBα level. AβO showed more potent effect on decreasing IκBα level than FAβ. On the other hand, FAβ and AβO significantly increased p65 level, with more increase in the AβO group. β-actin, as loading control, appeared similar band density among all groups.

4. Discussion

The present study showed that the chronic infusion of AβO or FAβ into the lateral ventricles of rats exhibited severe impairment on spatial learning and memory functions. The impairment induced by AβO was more severe than that by FAβ, and was accompanied by more neurodegeneration and more inflammatory response. Our results also evidenced that AβO possessed stronger ability in activation of NF-κB signaling system than FAβ.

It has long been assumed that Aβ has to be assembled into fibrillar amyloid plaques to exert its neurotoxic effects in AD. Accumulating data suggest that soluble Aβ oligomers induce neuronal dysfunction prior to the formation of fibrillar amyloid plaques. Thus, an alternative hypothesis is proposed: the soluble oligomers of Aβ play larger and earlier roles in neuronal damage than the insoluble components [17]. In *in vitro* experiments, while Aβ was assembled from oligomeric to fibrillar state, the ability to cause membrane permeation decreased [18]. The brain level of soluble Aβ species appeared to correlate better than the density of plaque deposition with the severity of cognitive impairment [19,20]. In this study, we infused FAβ or AβO into the lateral ventricles. After 30 days of chronic infusion, both types of Aβ1-42 induced AD-like changes and neurodegeneration in the hippocampus CA1 region. Compared to FAβ, AβO induced more impairment on cognitive functions and neuronal ultrastructure, indicating more neurotoxicity. The difference of neurotoxicity between FAβ and AβO in this study provides a direct evidence for the alternative hypothesis. More noticeably, AβO treatment disordered neurons array in the CA1 region. AβO also revealed great damage effect on organelles, especially mitochondria. These results further evidence the potent neurotoxicity of AβO.

As for the pathological mechanisms of AD involving Aβ, inflammation has been highlighted as one possible key contributing factor. The classic hallmarks of neuroinflammation, such as microglial activation, complement system activation and cytokine expression, are all observed in the brain tissues of AD patients [21]. The innate immune receptor TLR4, localized on the surface of microglia, is a first-line host defense receptor against invading microorganisms [22]. TLR4 was upregulated in brain tissues by Aβ1-42 aggregation,

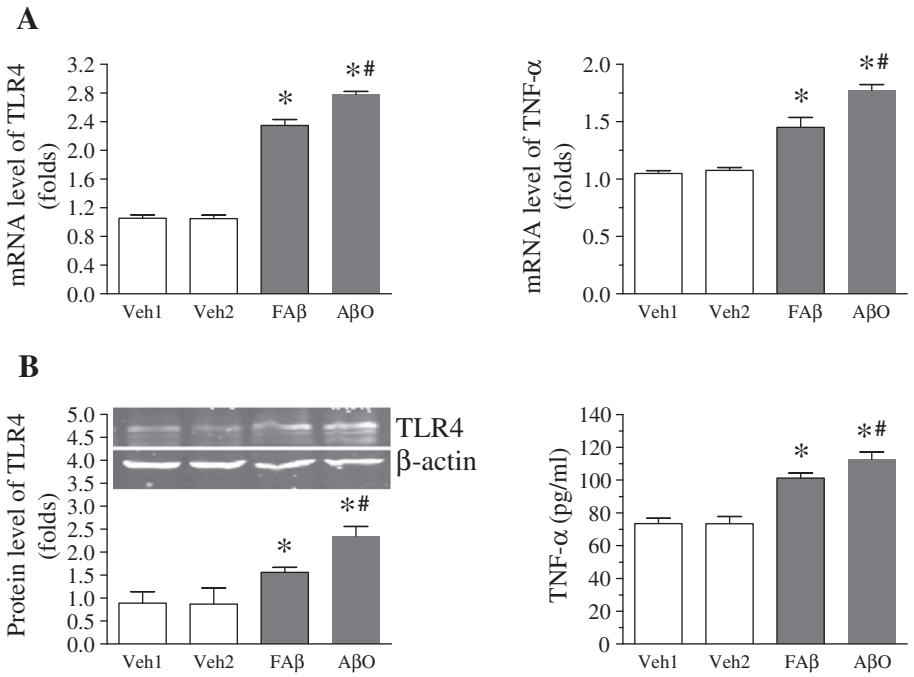

Fig. 3. mRNA and protein expressions of TLR4 and TNF-α induced by FAβ and AβO. The rats were sacrificed after MWM, and the hippocampal tissue was extracted to examine mRNA expressions of TLR4 and TNF-α with real time PCR (Fig. 3A), and protein expressions of TLR4 and TNF-α with western blotting and ELISA, respectively (Fig. 3B). The mRNA results were expressed as the ratio of TLR4 or TNF-α to β-actin. The Western blotting results were expressed as the ratio of optical density of TLR4 bands to β-actin bands. All values were mean ± SEM. ($n = 6$). $^{*}p < 0.05$ vs. the vehicle 1 (Veh1) or vehicle 2 (Veh2) group. $^{\#}p < 0.05$ vs. the FAβ group.

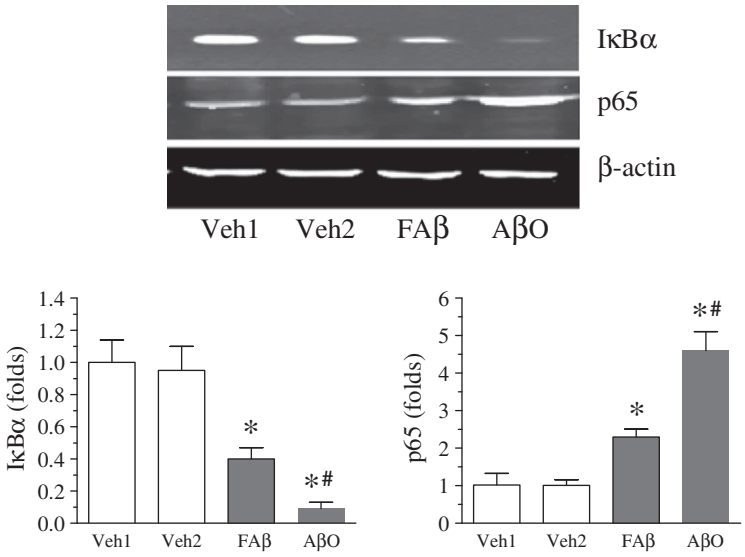

Fig. 4. NF-κB activation induced by FAβ and AβO. The rats were sacrificed after MWM, and the whole hippocampal tissue lysate was extracted to examine the levels of IκBα and p65 with western blotting. The results were expressed as the ratio of optical density of Iκbα or p65 bands to β-actin bands. All values were mean ± SEM. ($n = 6$). $^{*}p < 0.05$ vs. the vehicle 1 (Veh1) or vehicle 2 (Veh2) group. $^{\#}p < 0.05$ vs. the FAβ group.

and the upregulated TLR4 was associated with exacerbation of learning and memory functions [23]. A spontaneous loss-of-function mutation in TLR4 gene strongly inhibited Aβ aggregation-stimulated microglial and monocytic activation, and reduced the release of the inflammatory products TNF-α, IL-6 and nitric oxide [22]. In the present study, *we, at the first time, found that FAβ and AβO, like Aβ aggregation, stimulated TLR-4 expression.* Both FAβ and AβO also stimulated the expressions of cytokine TNF-α. In comparison with FAβ, AβO stimulated higher expressions of TLR4 and TNF-α. *The upregulation of TLR-4 and TNF-α by FAβ or AβO may exert pro-inflammatory effects which damage organelles and finally lead to neurodegeneration.* Thus, these data suggest that inflammation play an important role in Aβ-induced damage, and that the more toxic effect of AβO be related to the stronger inflammatory response.

The expression of inflammatory factors is involved in the activation of a variety of transcriptional factors, including NF-κB. NF-κB is activated by Aβ in many kinds of models. In macrophages and BV2 microglial cells, fibrillar Aβ induced IκBα phosphorylation and NF-κB activation, and then stimulated pro-inflammatory response, including the production of TNF-α, IL-6 and IL-1β [6,24]. AβO stimulated the production of TNF-α, IL-1β and cyclooxygen-

ase-2 in astrocytes *in vivo*, which was also medicated by NF-κB activation [16]. These previous reports reveal the importance of NF-κB in Aβ neurotoxicity. In this study, our results showed that both FAβ and AβO promoted IκBα degradation, and increased NF-κB p65 level. Compared to FAβ, AβO induced more degradation of IκBα and more increase of p65 level, which demonstrated more NF-κB activation in AβO treatment. NF-κB activation, together with the expression of TLR4 and TNF-α and the impairment of learning and memory functions, described a neurotoxicity pathway of FAβ and AβO from transcription, inflammatory response through function impairment.

Besides NF-κB-mediated inflammatory response, other mechanisms are involved in Aβ1-42-induced neural damage, such as glutamate receptor excitation [25], intracellular calcium and iron homeostasis disruption [26,27] and oxidative stress [27]. Therefore, the exploration for differences between FAβ and AβO in these aspects may contribute to further understanding of pathological mechanism of Aβ in AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.129.

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