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Atorvastatin Attenuates Cognitive Deficits and Neuroinflammation Induced by $A\beta_{1-42}$ Involving Modulation of TLR4/TRAF6/NF-κB Pathway

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Abstract

Inflammatory damage aggravates the progression of Alzheimer's disease (AD) and the mechanism of inflammatory damage may provide a new therapeutic window for the treatment of AD. Toll-like receptor 4 (TLR4)-mediated signaling can regulate the inflammatory process. However, changes in TLR4 signaling pathway induced by beta-amyloid (Aβ) have not been well characterized in brain, especially in the hippocampus. In the present study, we explored the changes of TLR4 signaling pathway induced by Aβ in the hippocampus and the role of atorvastatin in modulating this signal pathway and neurotoxicity induced by Aβ. Experimental AD rats were induced by intrahippocampal injection of $A\beta_{1-42}$, and the rats were treated with atorvastatin by oral gavage from 3 weeks before to 6 days after injections of $A\beta_{1-42}$. To determine the spatial learning and memory ability of rats in the AD models, Morris water maze (MWM) was performed. The expression of the glial fibrillary acidic protein (GFAP), ionized calcium binding adapter molecule-1 (Iba-1), TLR4, tumor necrosis factor receptor-associated factor 6 (TRAF6), and nuclear transcription factor (NF)-κB (NF-κB) protein in the hippocampus was detected by immunohistochemistry and Western blot. Compared to the control group, increased expression of TLR4, TRAF6, and NF-κB was observed in the hippocampus at 7 days post-injection of Aβ ($P < 0.01$). Furthermore, atorvastatin treatment significantly ameliorated cognitive deficits of rats, attenuated microglia and astrocyte activation, inhibited apoptosis, and down-regulated the expression of TLR4, TRAF6, and NF-κB, both at the mRNA and protein levels ($P < 0.01$). TLR4 signaling pathway is thus actively involved in Aβ-induced neuroinflammation and atorvastatin treatment can exert the therapeutic benefits for AD via the TLR4 signaling pathway.

Keywords Atorvastatin · Alzheimer's disease · Protection · TLR4 · TRAF6 · NF-κB

Introduction

In Alzheimer's disease (AD), accumulation of the beta-amyloid (Aβ) fibrils lead to neuroinflammation followed by oxidative stress and neuronal physiological changes that result in tangles, synaptic dysfunction, and neuronal loss (Bronzuoli et al. 2016). It is assumed that the neuroinflammation which modulates disease progression is a prominent trait of brain tissue in AD patients (Phillips et al. 2014; Schmöle et al. 2015). Accumulating evidence reveals that Aβ peptide aggregation can cause the activation of surrounding glial cells, and then the activated glial cells can initiate a neuroinflammatory response which involve the production of inflammatory mediators and the dysregulation of signaling pathways (Lv et al. 2014; Medeiros and LaFerla 2013).

Toll-like receptor 4 (TLR4) signaling pathway can sense the signal of pathogenic microorganisms invasion and tissue injury, and it also plays a vital part in the initiation of inflammatory responses (Calvo-Rodríguez et al. 2017), which contributes to neuroinflammatory injury in neurodegenerative diseases (Trotta et al. 2014). In AD patients, high expression of CD14 (receptor for TLR2 and TLR4) was observed in parenchymal microglia of the frontal and occipital neocortex, hippocampus, and around senile plaques (Letiembre et al. 2009; Liu et al. 2005). A murine AD model lacking the *CD14* gene had decreased Aβ plaque levels, altered inflammatory status of the brain, and reduced microgliosis (Reed-Geaghan et al. 2010). In vitro, the signal transduction cascades

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triggered by fibrillar Aβ are similar to that triggered by TLR agonists. TLR2, TLR4, and CD14 are required for the induction of nuclear transcription factor (NF)-κB dependent genes by Aβ (Costello et al. 2015). These evidences indicated that TLR plays a vital part in sensing and responding to Aβ (Carty and Bowie 2011). However, the changes in TLR4 signaling induced by Aβ in the brain, particularly in the hippocampus, have not yet been established. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an important effector among TLR downstream pathways. Studies have shown that TRAF6 can initiate the activation of nuclear factor-kappa B/p65 (NF-κB) and promote the translocation of NF-κB from cytoplasm to nucleus (Seok et al. 2015; Song et al. 2016). Activated NF-κB can increase the expression level of leukocyte adhesion molecules and pro-inflammatory cytokines, in turn promoting inflammation (Pal et al. 2016). However, so far, little is known regarding the expression of TRAF6 in the brain of AD patients.

Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are the most effective cholesterol-lowering agents among all hypolipidemic drugs (Geifman et al. 2017). Accumulating clinical epidemiological data has shown that the risk of developing incident dementia, mild cognitive impairment, and less cognitive decline in statin users was decreased compared to nonusers (Chou et al. 2014; Smith et al. 2017; Zissimopoulos et al. 2017). To elucidate the neuroprotection function of statins, the pleiotropic effects independent of cholesterol-lowering actions have been investigated, inclusive of anti-inflammatory, anti-oxidative stress, protection of the neurovascular unit, and facilitating exogenous Aβ degradation (Barone et al. 2014; Kurata et al. 2012a, b; Yamamoto et al. 2016). Compared to other statins, atorvastatin, one lipophilic member of statins family, has shown potential therapeutic anti-inflammatory effects (Zhao et al. 2016). However, the underlying mechanism of the neuroprotection function for atorvastatin applied to AD is not clear.

In the current study, we explored the changes in TLR4/TRAF6/NF-κB pathway induced by Aβ in rat hippocampus, the neuroprotection function of atorvastatin, and further evaluated whether rational modulation of this pathway could protect against Aβ-induced neurotoxicity.

Materials and Methods

Animals

Sprague-Dawley male rats, aged between 7 and 8 weeks, and weighing 250–300 g were used in the present study. They were obtained from Hebei Medical University. The protocol was approved by the Research Review Committee of The Second Hospital of Hebei Medical University. All rats were housed in the 12-h light and 12-h dark conditions in which the humidity was 60% ± 5% and the temperature was 22 ± 3 °C. All rats had free access to food and water.

Preparation of Amyloid-Beta Peptide and Surgical Intervention

The full length $A\beta_{1–42}$ (Sigma-Aldrich, Shanghai, China) was suspended in phosphate-buffered saline (PBS) (pH 7.4) at a concentration of 1 μg/μl. Before administration of $A\beta_{1–42}$, the suspended $A\beta_{1–42}$ was incubated at 37 °C with continuous stirring for 7 days to aggregate the $A\beta_{1–42}$ as described previously (Russo et al. 2012; Zhou et al. 2011). Chloral hydrate (300 mg/kg, ip) was used to anesthetize the rats, and then the rats were fixed on a stereotaxic apparatus. Both hippocampal CA1 regions were chosen for injection of $A\beta_{1–42}$. Sites were checked prior to injection of the methylene blue solution. Injection coordinates were chosen according to the atlas described by Paxinos and Watson (2005): anterior–posterior (AP): − 3.0 mm, medial–lateral (ML): 2.0 mm, and dorsoventral (DV): − 2.8 mm, according to bregma (Quan et al. 2013). The 10-μl aggregated $A\beta_{1–42}$ was injected slowly over 10 min (1 μl/min) using microsyringe with a stainless steel needle and syringe was maintained for 5 min at the injection site following the injection. Equal volume of PBS was injected into bilateral hippocampus of rats in the control group.

Experimental Groups and Drug Administration

We divided all rats into four groups as follows: (1) control group in which the rats were injected with PBS and oral saline into bilateral intrahippocampal; (2) the AD model group in which the rats were injected $A\beta_{1–42}$ and oral saline into bilateral intrahippocampal; (3) the atorvastatin (low concentration and high concentration)-treated groups (At-L and At-H) in which the rats were treated with injections of $A\beta_{1–42}$ and chronic administration of 5- and 10-mg/kg atorvastatin which suspended in sterile normal saline per day from 3 weeks before to 6 days after injections of $A\beta_{1–42}$. Atorvastatin (Lipitor, Pfizer-Parke Davis, Ireland) and saline were administered to rats by oral gavage. We examined the rats every day during the study, and the rats were weighed at intervals throughout the study. According to the previous studies (Zhang et al. 2014; Piermartiri et al. 2010), we determined the dose of the atorvastatin used in our study. This atorvastatin dose was consistent with the pharmacokinetic data that the rodents have high drug metabolic rate (Dostal et al. 1996), even though the dose was higher compared to the recommended does (1.1 mg/kg per day) for treatment of hypercholesterolemia in human.

Morris Water Maze Test

Morris water maze (MWM) task was performed according to previously described (Xie et al. 2012) on 7 days post-injection

of $A\beta_{1-42}$ (the rats for behavioral tests, $n = 6$ per group). A tank was acted as a maze. And the diameter and the height of the maze were 180 and 70 cm, respectively. The maze was filled with water at 22 ± 1 °C. There were four quadrants in the tank. A circular escape platform with 10 cm of diameter was only placed in one quadrant. The fixed position of the escape platform was 2 cm under the water. The rats were accepted training for 5 days with two sessions every day and an inter-session interval of 2 h. There were four trials and 30 s of inter-trial interval each session. In each trial, we placed the rats gently in one quadrant randomly with its nose pointing toward the wall and allowed them to find the escape platform. We recorded the time of escape latency as 120 s if a rat did not find the platform within 120 s, and then the rat was placed on the circular platform. The rats were kept for 10 s on the platform before the start of the next trial. To determine the ability of spatial learning, the time of the rat spent to reach the platform (escape latency) was recorded. On the sixth day, a probe trial of spatial memory was conducted by removing the platform and measuring the time spent in the target quadrant and the number of crossings over the former platform location. To minimize the performance—differences caused by circadian rhythmicity, the MWM test was performed between 9:00 a.m. and 18:00 p.m.

Immunohistochemistry

On 7 days after injection of $A\beta_{1-42}$, 4% paraformaldehyde was used to fix the rat brains for 24 h, and then the fixed brains were paraffin embedded. To conduct the hematoxylin-eosin (HE) staining and immunohistochemistry, 5-μm sections were made, and then the specimens were incubated with rabbit anti-ionized calcium binding adapter molecule-1 (Iba-1) antibody (dilution, 1:500; Wako Chemicals, Richmond, VA), anti-glial fibrillary acidic protein (GFAP) antibody (dilution, 1:1000; Millipore), anti-NF-κB and anti-TRAF6 rabbit polyclonal antibody (dilution, 1:100, Santa Cruz Biotechnology), and anti-TLR4 mouse monoclonal antibody (dilution, 1:50, Santa Cruz Biotechnology) at 4 °C overnight. SP rabbit/mouse HRP kit (DAB) (Co-win Biology Technology Company, China) contained the secondary antibodies, secondary biotinylated conjugates, and diaminobezidine. To count the Iba-1-positive, TLR4-positive, TRAF6-positive, and NF-κB-positive cells throughout four fields in the CA1 region of hippocampal, an examiner blinded to the experimental groups performed the cell count under a ×400 light microscope. Average optical density (AOD) of GFAP immunoreactive intensity was calculated using Image-Pro Plus 6.0 Analysis System (Media Cybernetics, Rockville, MD, USA). Each group was taken five rats and each rat was taken three coronal hippocampal sections (Ryu et al. 2009).

Western Blot

Total Protein Extraction Kit (Applygen Technologies Inc., Beijing) was chosen to extract the total protein from hippocampus of rats according to the manufacturer's protocols. BCA Protein Assay reagent kit (Novagen, Madison, WI, USA) was used to determine the concentrations of extracted protein. SDS-PAGE was performed to separate equal amounts of proteins ($n = 6$ in each group) per lane, and then the proteins were transferred to PVDF membranes. PVDF membranes were blocked for 2 h at room temperature using the blocking buffer (Tris-buffered saline, 0.1% tween-20 with 5% *w*/*v* non-fat dry milk) and then were incubated with anti-cleaved caspase-3 antibody (dilution, 1:1000; Cell Signaling Technology), anti-TLR4 antibody (dilution, 1:1000; Bioworld), anti-TRAF6 antibody (dilution, 1:500; Santa Cruz), anti-NF-κB antibody (dilution, 1:2000; Santa Cruz), anti-β-actin antibody (dilution, 1:5000; Zhongshan Biotechnology), and anti-histone H3 antibody (dilution, 1:1000; Bioworld, Louis Park, MN, USA) overnight at 4 °C. Membranes were washed with 0.1% tween-20 Tris-buffered saline after the incubation of primary antibodies and then were incubated with second antibodies (goat anti-rabbit, 1:6000, Rockland, Gilbertsville, PA or goat anti-mouse, 1:3000, Rockland, Gilbertsville, PA) for 1 h at room temperature. The relative densities of the blots were analyzed by imaging densitometer (LI-COR Bioscience). In the present study, β-actin was used as internal control for cytoplasmic protein and histone H3 as internal control for nuclear protein.

Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from hippocampal tissue using the TRIzol reagent (ThermoFisher Scientific, Shanghai, China) according to the manufacturer's recommendations. Samples were reverse transcribed using the first-strand cDNA synthesis kit (Fermentas International Inc., Burlington, Canada). Obtained cDNA was amplified by a real-time PCR system (Agilent, USA) in the presence of a fluorescent dye (SYBR GreenI; Cwbio). The primers used are presented as follows:

Tlr4: Forward 5′-GAATGAGGACTGGGTGAGAAAC-3′,
Reverse 5′-CTCAGCAAGGACTTCTCCACTT-3′;
Traf6: Forward 5′-TGGATTCTACACAGGCAGACC-3′,
Reverse 5′-TCAAAGCGGGTAGAGACTTCA-3′;
Gapdh: Forward 5′-TGAACGGGAAGCTCACTGG-3′,
Reverse 5′-GCTTCACCACCTTCTTGATGTC-3′.

All data were subsequently normalized to *Gapdh* mRNA level. The relative quantification of mRNA expression was calculated according to the $2^{-\Delta\Delta Ct}$ method ($n = 3$ per group).

Statistical Analysis

The SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used to conduct the statistical analysis. Statistical analysis of data were performed by one-way ANOVA and followed by Student-Newman-Keuls test for intergroup comparisons. Two-way repeated factor analysis of variance (ANOVA) with Student-Newman-Keuls tests was conducted to analyze the escape latency during the training tests. $P < 0.05$ was considered statistically significant.

Result

Atorvastatin Ameliorated the Impairments of Spatial Learning Ability and Memory

As shown in Fig. 1, MWM test was performed to analyze the effect of atorvastatin in AD rats induced $A\beta_{1-42}$. Rats in AD group showed significantly longer escape latencies compared to that of rats in the control group ($P < 0.01$), which indicated that the $A\beta_{1-42}$ induced learning impairment of rats.. However, the poor performance was alleviated after the high-dose atorvastatin treatment ($P < 0.05$, Fig. 1a). Consistently, compared to the control group, in the probe trials, the duration in the target quadrant or the number of crossings over the platform location was significantly reduced in the AD group, while treatment with high-dose atorvastatin improved the performance ($P < 0.01$, Fig. 1). In addition, compared to the AD group, the rats in the At-L group presented the decreased escape latencies, longer time in the target quadrant, and increased number of crossings, while these differences were not statistically significantly ($P > 0.05$).

Atorvastatin Improved Pathological Changes and Cell Apoptosis in Rats' Hippocampus

As shown in Fig. 2a, in the control group, the pyramidal cells in the CA1 region were arranged neatly and tightly, and little cell loss was observed. However, after $A\beta_{1-42}$ injections, the cell number in the CA1 region was decreased and some cells were arranged irregularly. Furthermore, the degenerated neurons which showed membrane shrinkage, nucleus pyknotic, and intensive blue-stain in cell body were found (Fig. 2b), and the percent of degenerated neurons was significantly increased ($P < 0.01$, 2E). These abnormalities of pyramidal cells were attenuated by high, and not low, dose of atorvastatin treatment (Fig. 2c–e). To determine apoptosis, we evaluated the level of activated (cleaved) caspase-3 protein which is a critical executioner and early marker of apoptosis. In agreement with the results of HE staining, the level of cleaved caspase-3 was significantly increased at 7 days after $A\beta_{1-42}$ injection compared with the control group ($P < 0.01$), and treatment with high-dose atorvastatin significantly prevented the $A\beta_{1-42}$ induced caspase-3 activation (Fig. 2f and g, $P < 0.01$).

Atorvastatin Reduced Microglia and Astrocyte Activation

$A\beta_{1-42}$ injections significantly activated astrocytes and microglia in CA1 regions of rat's hippocampal. The number of Iba-1-positive microglia and GFAP-positive astrocytes increased (Fig. 3, $P < 0.01$). The number of GFAP-positive astrocytes was detected by measuring the AOD of GFAP expression. High-dose atorvastatin treatment significantly prevented the activation of microglia and astrocytes induced by $A\beta_{1-42}$ in rat hippocampal ($P < 0.01$), while there was no

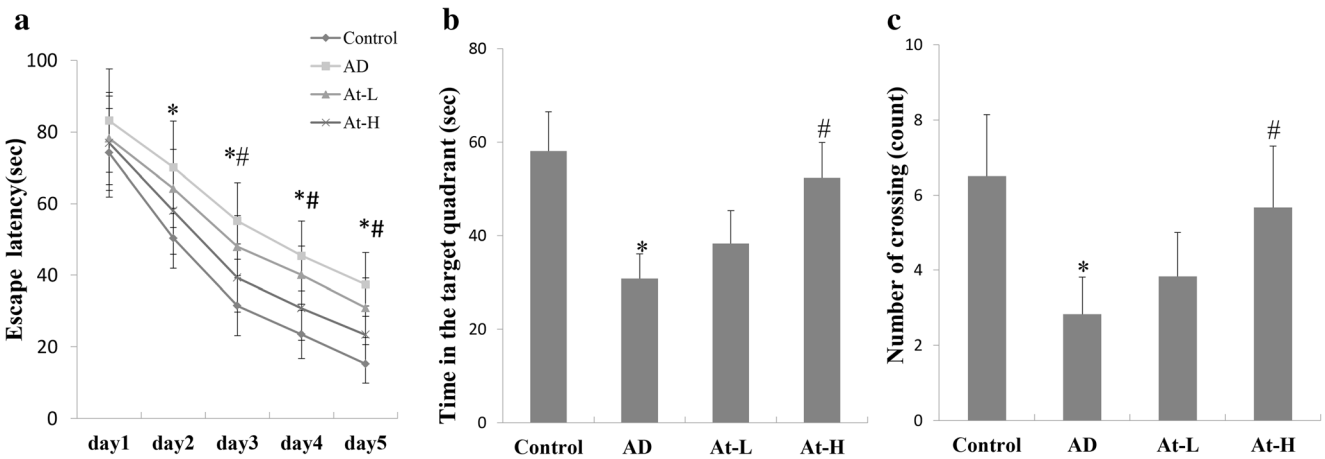

Fig. 1 Atorvastatin improved the impairment of spatial learning and memory of rats induced by $A\beta_{1-42}$, using the Morris water maze test. The "Control," "AD," "At-L," and "At-H" represent the control, AD model, intrahippocampal injections of $A\beta_{1-42}$ and oral atorvastatin (5 mg/kg), and intrahippocampal injections of $A\beta_{1-42}$ and oral atorvastatin (10 mg/kg), respectively. The rats in the control group received bilateral intrahippocampal injection of PBS and oral saline. Latencies to reach escape platform (**a**). Time spent in target quadrant (**b**). The number of crossing the platform (**c**). Values are expressed as mean ± SD (*$P < 0.01$ compared with the control group; #$P < 0.01$ compared with the AD group)

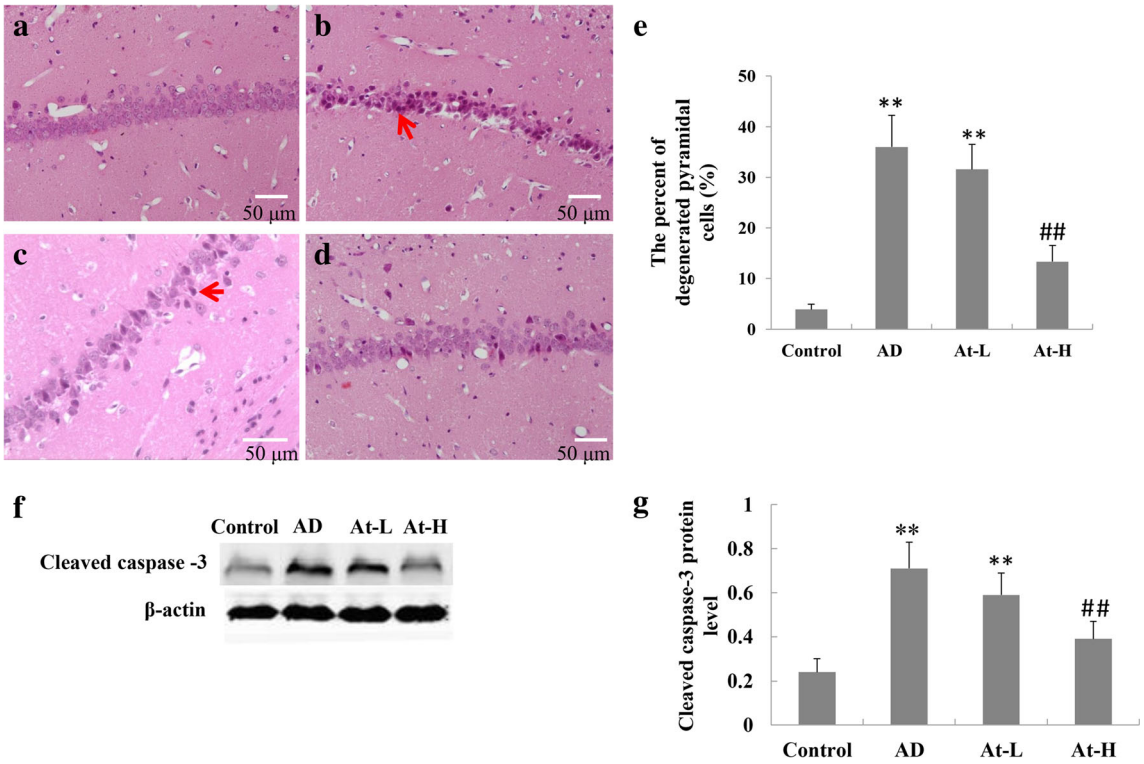

Fig. 2 Pathological changes and cell apoptosis in rats' hippocampus determined by HE staining (×400). **a** Control group. **b** AD model group. **c** At-L group. **d** At-H group and Western blotting (**f**, **g**). Rats in the control group did not show histopathological abnormalities. In the AD and At-L groups, the proportion of degenerated neurons with membrane shrinkage, nucleus pyknotic, and intensive blue-stain in cell body (arrowheads) increased in the hippocampal CA1 region compared with the control group (**e**). Furthermore, the remnants of the pyramidal cells were arranged irregularly and some exhibited shrunken and the number of cells appeared decreased. The cells in the At-H group had better cell morphology and were more numerous than those in the AD groups. Apoptosis was determined by cleaved caspase-3 using Western blot (**f**, **g**). ** represents $P < 0.01$ vs. control group. # represents $P < 0.05$ vs. AD group. ## represents $P < 0.01$ vs. AD group

significant decrease in the activation of microglia and astrocytes after low-dose atorvastatin treatment.

Atorvastatin Suppressed the Immunoreactivity of TLR4, TRAF6, and NF-κB

The immunoreactivity of TLR4, TRAF6, and NF-κB protein in the hippocampal CA1 regions was identified by immunohistochemistry on day 7 after $A\beta_{1–42}$ injections. The results presented that in control group, few cells were TLR4-positive, TRAF6-positive, and NF-κB-positive in the CA1 region. In the AD group, the number of TLR4-positive, TRAF6-positive, and NF-κB-positive cells was significantly higher than that in the control group and the location of NF-κB was mostly in nucleus. In addition, on the 7 day after $A\beta_{1–42}$ injection, the immunoreactivity of TLR4 was high in both pyramidal and glial cells of CA1 region. Compared to the AD group, the numbers of TLR4-positive, TRAF6-positive, and NF-κB-positive cells significantly decreased in At-H group. Moreover, the cells positive for nuclear NF-κB staining were also reduced. However, no significant difference was found in the positive cell number between AD group and At-L group (Fig. 4).

Atorvastatin Decreased the Expression of TLR4, TRAF6 Protein Levels, and Nuclear Translocation of NF-κB

Western blot was performed to further determine the protein levels of total TLR4, TRAF6, and nuclear NF-κB. In control group, the expression level of NF-κB p65 was high in cytosolic and low in nuclear. In AD group, the level of nuclear NF-κB in hippocampus significantly increased, whereas its level in cytosolic concurrently decreased. These results indicated that the NF-κB subunits translocated to nucleus from the cytosol. In agreement with the immunohistochemistry results, Western blot analysis revealed that the expression levels of TLR4, TRAF6, and nuclear NF-κB protein upregulated in AD group ($P < 0.05$). The mRNA expression of *Tlr4* and *Traf6* was increased in AD group, compared with control group ($P < 0.05$). The over-expression of TLR4, TRAF6, and nuclear NF-κB protein induced by $A\beta_{1–42}$ in the hippocampus was decreased after high-dose atorvastatin treatment ($P < 0.05$ Fig. 5). However, no significant differences were observed in the expression of TLR4, TRAF6, and nuclear NF-κB p65 between AD group and At-L group ($P > 0.05$).

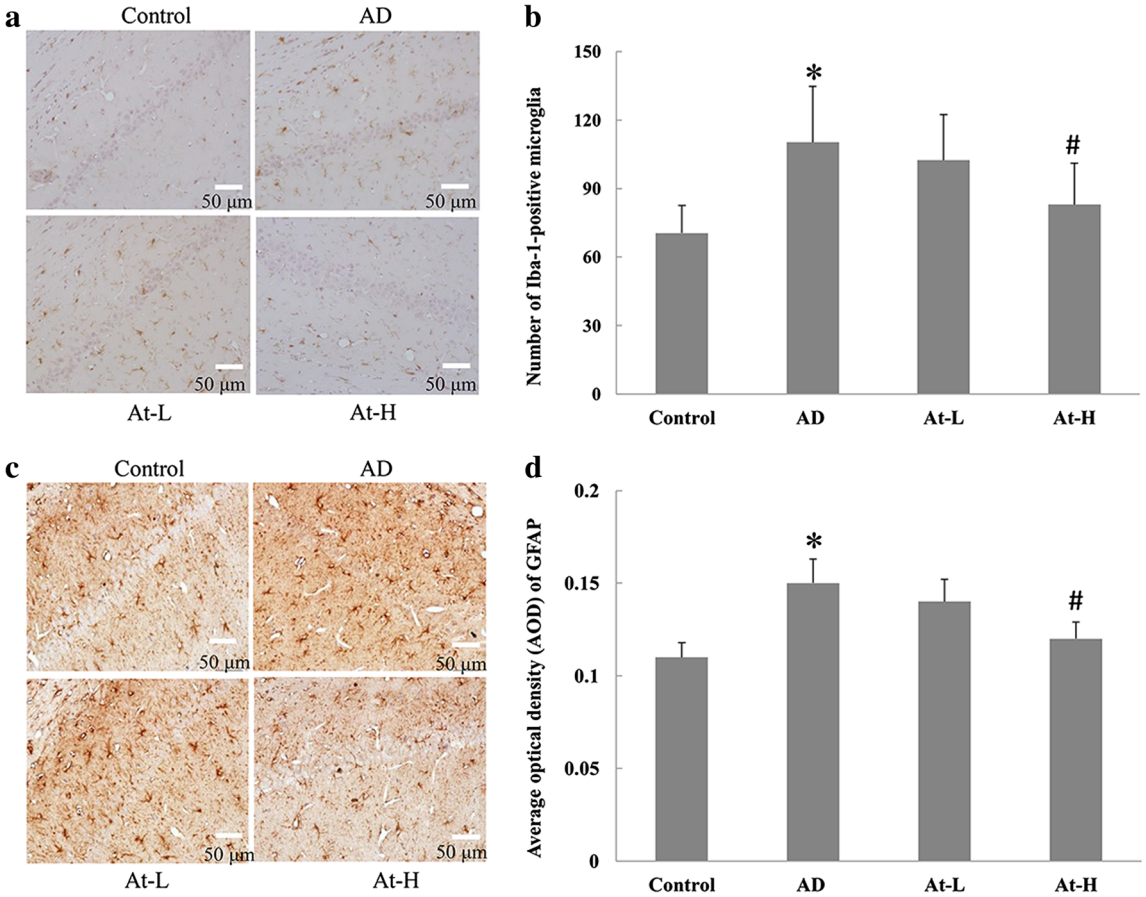

Fig. 3 Atorvastatin reduced microglia and astrocyte activation. Effects of atorvastatin on the $A\beta_{1-42}$ induced increase of Iba-1-positive microglia (**a**, **b**) and GFAP-positive astrocytes (**c**, **d**) in the hippocampal CA1 region. Photomicrographs are representative of immunohistochemical hippocampal CA1 regions (×400 magnification). Bar graphs illustrate the number of Iba-1-positive microglia (**b**) and the AOD of GFAP expression (**d**), respectively. Values are expressed as means ± SD ($n = 5$/group). * $P < 0.01$ compared with the control group; # $P < 0.01$ compared with the AD group

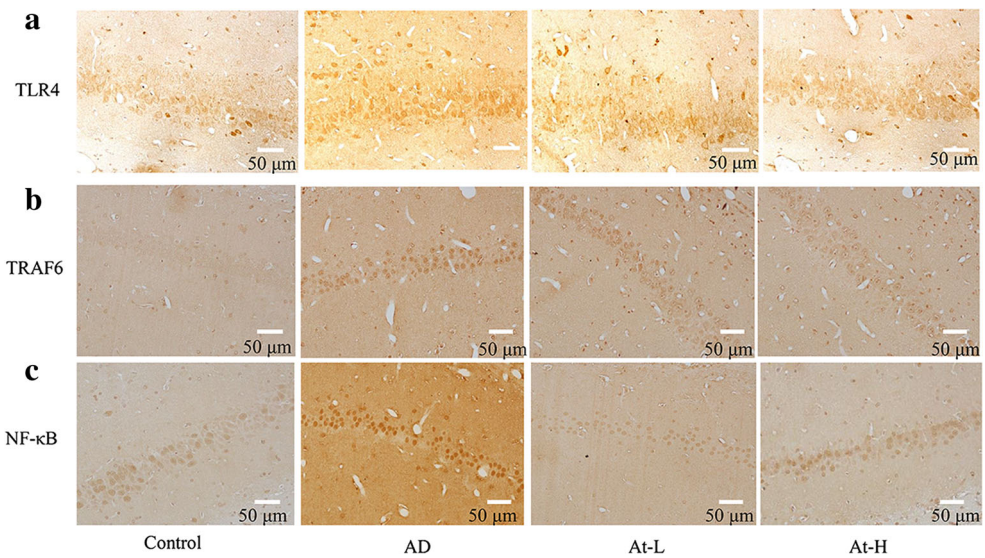

Fig. 4 Protein expression of TLR4, TRAF6, NF-κB in rat hippocampus examined by immunohistochemical staining of (**a**) TLR4, (**b**) TRAF6, and (**c**) NF-κB in the hippocampal CA1 region at 7 day post-injection of $A\beta_{1-42}$ (×400 magnification). There was increased TLR4, TRAF6, and NF-κB immunoreactivity after $A\beta_{1-42}$ injections. Atorvastatin administration reduced TLR4, TRAF6, and NF-κB immunoreactivity in CA1 region, which was most marked in the At-H group

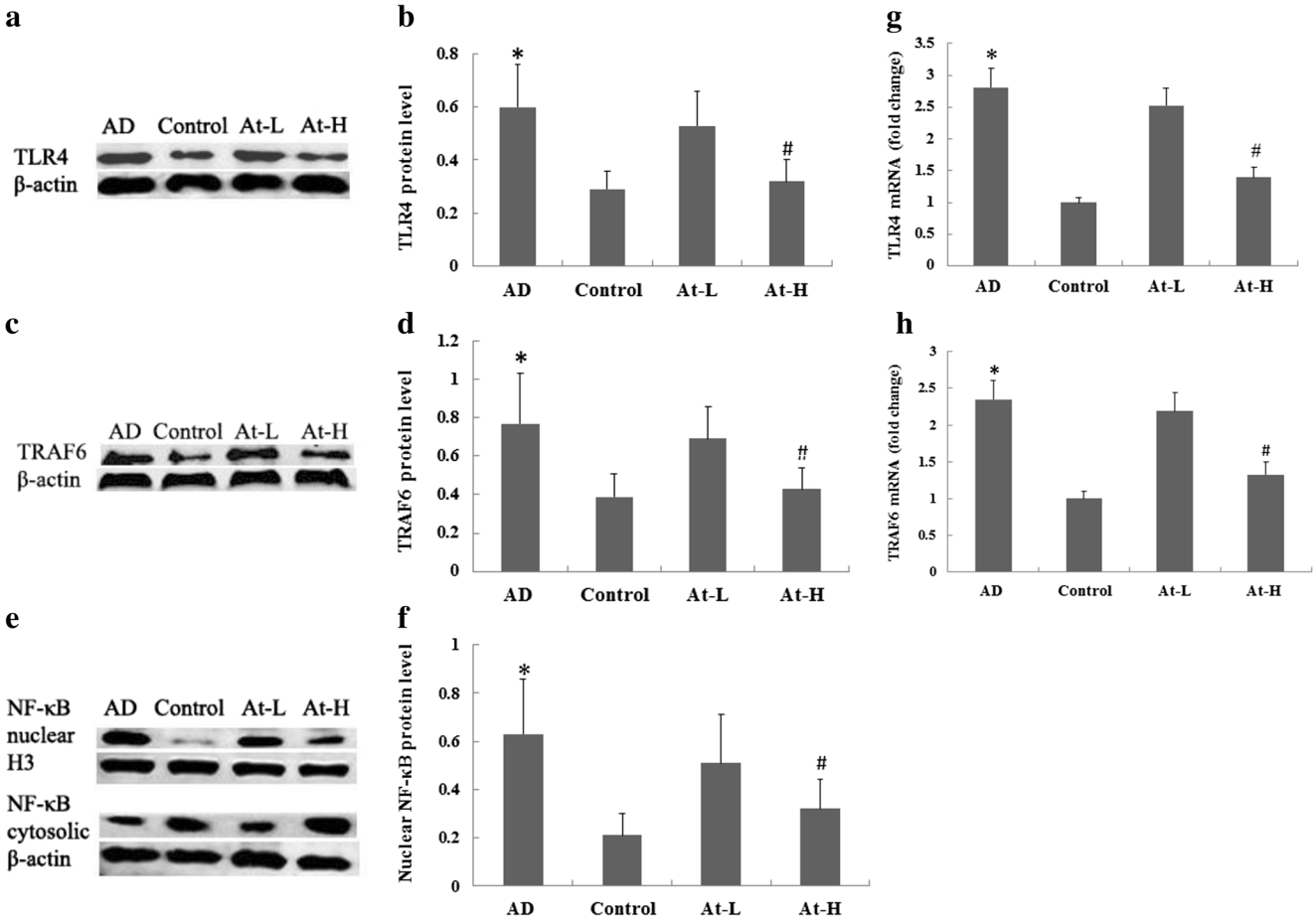

Fig. 5 Protein and mRNA expression of TLR4, TRAF6, and NF-κB in rat hippocampus. Compared with control group, the expression of total protein and mRNA of TLR4 (protein—**a**, **b**; mRNA—**g**), TRAF6 (protein—**c**, **d**; mRNA—**h**), and nuclear protein of NF-κB (**e**, **f**) was significantly increased at 7 days post-injection of $A\beta_{1–42}$.* $P < 0.05$vs. control group. The increased expression in TLR4 (**a**, **b**, **g**), TRAF6 (**c**, **d**, **h**), and nuclear NF-κB (**e**, **f**) protein levels induced by $A\beta_{1–42}$ was prevented in the At-H group. # $P < 0.05$ vs. AD group

Discussion

The prominent characteristic of AD is neuroinflammation. The glial cells can be activated by Aβ deposition. The activated glial cells can release a wide spectrum of cytokines and chemokines which lead to oxidative stress, synaptic dysfunction, and neuronal loss (Capiralla et al. 2012). Anti-inflammation therapies have generated considerable interest to combat Aβ-induced damage (Doost et al. 2015). Studies have suggested the $A\beta_{1–42}$ injection model can have particular utility in exploring the role of inflammation in AD (McLarnon and Ryu 2008; Xuan et al. 2012).

Clinical data has revealed that statins might decrease the AD incidence rates (Chou et al. 2014). Meanwhile, study showed that the primary role of statins for AD patients was reducing inflammation response which was induced by the activation of microglia instead of decreasing the accumulation of Aβ (Wolozin 2004). The statins can reduce the inflammation mediated by Aβ42 peptides, even though the timing of statin treatment remains poorly established (Griffin et al. 2016). The increased level of anti-inflammatory cytokine interlrukin-4 (IL-4) and decreased intracellular expressions of TNF-α, IL-6, and IL-1β induced by Aβ in the hippocampus were observed after pre-treatment with atorvastatin for 3 weeks (Clarke et al. 2007; Zhang et al. 2013). In our study, results presented that chronic treatment with high-dose atorvastatin (10 mg/kg/day, from 3 weeks before to 6 days after injections of $A\beta_{1–42}$) can significantly prevent $A\beta_{1–42}$-induced activation of microglia and astrocytes at 7 days post-injection of $A\beta_{1–42}$ into rat hippocampus. This intervention simultaneously attenuated the impairment of learning ability and memory of rats, pathological changes, and apoptosis induced by Aβ in rats' hippocampus. These results indicate that atorvastatin might improve learning and memory ability of the AD rat models by inhibiting inflammatory response. However, previous study has reported that 1 week of atorvastatin treatment (10 mg/kg/day) failed to improve the cognitive deficits of $A\beta_{1–40}$-treated mice even though atorvastatin

treatment reduced oxidative stress and inflammatory responses induced by $A\beta_{1-40}$ (Piermartiri et al. 2010). Our results also revealed that continuous administration of low-dose atorvastatin had the trend to improve the Aβ-induced cognitive impairment compared with control group, while this difference was not significant, which implied that the neuroprotective effects of atorvastatin in AD seemed to be dependent on the dose and schedule of treatment utilized.

Of note, we did not test the toxicity of atorvastatin because the dose used in our study has been used in several previous studies. A spate study had reported that the decrease of synaptic density in the hippocampus was not observed in rats within the "sham + atorvastatin" group chronically administrated with atorvastatin (10 mg kg^{-1} day^{-1}, po) compared to the "sham" group (Zhang et al. 2014). Similarly, it was shown that the administration of atorvastatin (10 mg/kg, po) for 42 days could prevent retrograde amnesia of rats suffering chronic cerebral hypo-perfusion (Zaghi et al. 2016). Oral treatment with atorvastatin (10 mg/kg/day) improved cognitive impairments and increased hippocampal levels of nerve growth factor in an experimental rat model of Parkinson's disease, and no rats exhibited the toxicity of atorvastatin (Castro et al. 2013). The administration of atorvastatin in our study was consistent with another study in which the ameliorative roles of atorvastatin in a rat model of vascular dementia were examined (Koladiya et al. 2008). Furthermore, in our preliminary experiment, we chose some rats ($n = 6$) from control group and these rats were subjected to the same administration of atorvastatin. Based on these previous studies and our preliminary analyses (data not shown), we concluded that the dose of atorvastatin used in the study was safe for rats.

The pattern-recognition receptor TLRs can recognize conserved pathogen-associated molecular pattern. The innate immunity can be activated via TLR ligation after microbial infection. However, it is now obvious that TLRs can also identify endogenous signals including different components of the extracellular matrix, intracellular proteins, or lipoproteins and contribute to the initiation of the inflammatory response as well as cell apoptosis (Tang et al. 2008; Zhang et al. 2013b). Recent evidence in cell culture indicates several Toll-like receptors (TLRs), including TLR4, are primary receptors for Aβ to trigger neuroinflammatory activation, and inhibition of TLR4 signaling may protect against Aβ-mediated inflammation (Zhao et al. 2014; Capiralla et al. 2012). In addition, genetic association studies for TLR4 also suggested that the TLR4 polymorphisms were significantly related with the risk of late-onset AD (Balistreri et al. 2008). In our study, the TLR4 immunoreactivity was increased both in pyramidal neuron and glial cells of the hippocampus CA1 region on day 7 post-injection. TLR4 intracellular signaling pathways are transduced through myeloid differentiation primary-response protein 88(MyD88)-TRAF6. MyD88 recruitment initiates activation of interleukin receptor-associated kinase4 (IRAK4). The IRAK4 can phosphorylate IRAK1 and cause the recruitment of TRAF6 and TGF-β-activated kinase 1 (TAK1). These signaling molecules can induce nuclear translocation of NF-κB from cytoplasm to nuclear, which resulted in the production of inflammatory cytokines and chemokines (Shi et al. 2016). Now, little is known on the expression of TRAF6 and its role in the AD models. In our study, we observed that intrahippocampal delivery of $A\beta_{1-42}$ can induce the increased expressions of TLR4, TRAF6, and nuclear NF-κB p65 in vivo on the 7 day of post-injection, which indicated that TLR4/TRAF6 signaling may be involved in neuroinflammation triggered by Aβ.

Conflicting results were presented among the studies determining the effects of modulating the TLR4 signaling on AD progression. It was reported that TLR4 mutation increased Aβ deposition and exacerbated cognitive deficits in an AD transgenic model (Song et al. 2011). However, Reed-Geaghan et al. (2010) found that the loss of CD14 resulted in a significant change in the inflammatory environment of the brain and decreased plaque load at an intermediate stage of plaque deposition period. In AD murine model of which the *IRAK4* (downstream kinase of TLR4) was knocked out, microgliosis of brain was decreased and cognitive dysfunction was attenuated (Cameron et al. 2012). In our study, we found that high-dose atorvastatin significantly decreased the levels of TLR4, TRAF6, and NF-κB, reduced Aβ induced gliosis, and simultaneously ameliorated impairments of spatial learning ability and memory in Aβ-injected rat. These results from Aβ-injected rat model reinforced the notion that proper modification of TLR4 signaling might exert the therapeutic benefits for AD (Gambuzza et al. 2014).

NF-κB exists in almost all cells. Our result showed that Aβ activated TLR4-mediated signal transduction pathway which contributed to translocate NF-κB from cytoplasm into nucleus in the hippocampus. NF-κB combines with specific DNA sequence to stimulate downstream factors inducing inflammation response and neuronal apoptosis (Li et al. 2016; Zheng et al. 2014). The present results for the first time showed that high-dose atorvastatin significantly decreased the nuclear translocation of NF-κB induced by Aβ in the hippocampus of rats, which enriched its neuroprotective molecular effects.

One question that may arise is whether atorvastatin reduces inflammatory responses triggered by Aβ aggregates rather than reducing deposition of Aβ aggregates. Levels of Aβ in the brain had to be determined before atorvastatin treatment, before Aβ injection, and 6 days after the injection. Our major focus in this study was on Aβ aggregates triggering neuroinflammation, the potential cellular signaling pathway, and the mechanism underlying anti-inflammatory effects of atorvastatin. Little Aβ42 was detected in SD rat's hippocampus in a previous study (Quan et al. 2013). Moreover, short- or long-term treatment with atorvastatin in rats and dogs, respectively, had no effect on amyloid beta in brain or cerebrospinal fluid

(Cibickova et al. 2009; Murphy et al. 2010). Up to date, no study in vivo has shown that atorvastatin can affect the degradation of exogenous Aβ. Furthermore, the injection model could have utility in addressing points regarding the roles of chronic inflammation in AD and bypass effects of abnormalities in the processing of amyloid precursor protein (McLarnon and Ryu 2008). The model uses $A\beta_{1-42}$ peptide as an initiator of inflammatory responses and minimizes other critical factors. In most studies which were designed to assess Aβ injection inducing neurotoxicity and pharmacological modulation, levels of Aβ in the brain have not been determined, because of an acute exogenous Aβ infusion rather than abnormal chronic production of peptide in transgenic animal models of AD (Ryu and McLarnon 2008; Li et al. 2017; Tang et al. 2014; Choi et al. 2012; Lyons et al. 2011). So based on the results of previous studies, we did not determine levels of Aβ in the brain.

In conclusion, our findings indicated that the TLR4 signaling pathway was involved in Aβ-induced neuroinflammation in the hippocampus. Systemic administration of atorvastatin significantly decreased the expression level of TLR4, TRAF6, as well as nuclear NF-κB and attenuated cognitive deficits induced by the Aβ, the hippocampal pathological changes, neuronal apoptosis and the activation of microglia, and astrocytes in AD rat model. These results provide evidence for the potent anti-inflammatory activity of atorvastatin in AD brain tissue. At last, we suppose the proper modification of TLR4-mediated signaling pathways may be one of the effective therapeutic targets of atorvastatin for Aβ-induced neurotoxicity in AD.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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