Atorvastatin protects BV-2 mouse microglia and hippocampal neurons against oxygen-glucose deprivation-induced neuronal inflammatory injury by suppressing the TLR4/TRAF6/NF-κB pathway

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Abstract. Atorvastatin is a member of the statin class of drugs, which competitively inhibit the activity of 5-hydroxy-3-methylglutaryl-coenzyme A reductase. The aim of the present study was to assess whether atorvastatin may protect BV-2 microglia and hippocampal neurons against oxygen-glucose deprivation (OGD)-induced neuronal inflammatory injury and to determine the underlying mechanisms by which its effects are produced. Cell viability and apoptotic ability were assessed using an MTT assay and annexin V-fluorescein isothiocyanate/propidium iodide double staining followed by flow cytometry, respectively. The expression of inflammation and apoptosis-associated mRNAs and proteins were assessed using reverse transcription-quantitative polymerase chain reaction and western blotting, and the expression of inflammatory factors was determined using ELISA. The results of the current study revealed that atorvastatin treatment suppressed the viability of OGD BV-2 microglia and hippocampal neurons. Furthermore, atorvastatin treatment reduced the expression of proinflammatory factors in OGD BV-2 microglia. Additionally, it was demonstrated to downregulate the toll-like receptor 4 (TLR4)/tumor necrosis factor receptor-associated factor 6 (TRAF6)/nuclear factor-κB (NF-κB) pathway in OGD BV-2 microglia. Atorvastatin also inhibited the apoptosis of OGD hippocampal neurons by regulating the expression of apoptosis-associated proteins. It was concluded that atorvastatin treatment may protect BV-2 microglia and hippocampal neurons from OGD-induced neuronal inflammatory injury by suppressing the TLR4/TRAF6/NF-κB pathway. This may provide a potential strategy for the treatment of neuronal injury.

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Introduction

Inflammation is a complex defense mechanism that involves specialized cells and soluble factors, which are initiated to suppress and combat external threats to protect normal physiological functions (1). Analogous inflammatory progressions are considered to occur in the brain and peripheral tissues. In the brain, microglia act as important immune effector cells of the central nervous system (CNS); microglia are sensitive to external environmental stimuli and are the first cells to respond following damage (2). Under normal conditions, brain microglia are in a quiescent state. However, following ischemia and hypoxia, microglia rapidly transform from a resting to an active state, increasing their production of inflammatory cytokines (3). Chronic inflammation of the brain gradually becomes noxious, which is associated with progressive tissue damage in degenerative diseases.

Alzheimer's disease (AD) is a degenerative disease of the CNS, the pathological characteristics of which were first described by the German doctor Alios Alzheimer in 1906 (4). Among the various proposals for mechanisms underlying pathogenesis of AD, inflammation is a theory of interest. Previous studies have demonstrated the existence of inflammatory markers in AD brains, including cytokine and chemokine overproduction, and microgliosis within injured areas (5-8). Additionally, it has been demonstrated that levels of various proinflammatory factors were enhanced in patients with AD, including interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) (9). Activated microglia have been revealed to exert neurotoxic effects by releasing IL-6, IL-1β, TNF-α and inducible nitric oxide (NO) synthase (8), which is reported to be exacerbated in neurodegenerative diseases, including AD (10), human immunodeficiency virus-associated dementia (11) and Parkinson's disease (12).

Toll-like receptors (TLRs) are crucial innate immune receptors that have been reported to be involved in the development and progression of AD (13). Previous studies have

demonstrated that TLRs serve critical roles in anoxic and anoxemic injury (14-16). It has been demonstrated that TLR4 knockout protected against ischemic injury in mice (17). As one of the key inflammatory-associated molecules, TNF receptor-associated factor 6 (TRAF6) has been demonstrated to induce nuclear factor-κB (NF-κB) activation (18). NF-κB is a key transcription factor that participates in the regulation of inflammatory cytokine expression. NF-κB may be transported into the nucleus following TLR signaling pathway activation (19). Furthermore, it has been indicated that suppressing the activation of NF-κB is associated with neuroprotective effects (20,21). It has been demonstrated that the TLR4 signaling pathway may be implicated in the progression of AD and may therefore be a novel therapeutic target (22). However, the function and mechanism of the TLR4/TRAF6/NF-κB pathway in AD has not yet been elucidated.

Statins, a class of 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, were first isolated from the fungus *Penicillium citrinum* in 1976 (23). HMG-CoA reductase serves as a rate-limiting enzyme in the synthesis of cholesterol in hepatic cells, and its activity may be competitively suppressed by statins (24). In addition, a previous study demonstrated that statins serve an indispensable role in the protection of the CNS and the improvement of memory (25). Atorvastatin is a statin that, when applied long-term, has been revealed to improve learning and memory in AD rats (26). Atorvastatin has also been demonstrated to downregulate the expression of IL-6, IL-1β and TNF-α in the hippocampus, further protecting the CNS (27). However, there is a lack of research at present concerning atorvastatin and its protective effects against neuronal inflammatory injury in AD.

The present study assessed whether atorvastatin may have potential as a novel therapeutic agent in the suppression of proinflammatory and neurotoxic factor release from oxygen-glucose deprived (OGD) BV-2 microglia and the apoptosis of OGD hippocampal neurons. Furthermore, the associated signaling pathway and apoptosis-associated protein expression in OGD models treated with atorvastatin were assessed.

Materials and methods

Ethical approval. Ethical approval for the experimental research on animals in the present study was obtained from the Institutional Review Board of Shaoxing Municipal Hospital (Shaoxing, China).

Reagents. DMEM medium and penicillin-streptomycin used in cell culture were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following antibodies were purchased from Abcam (Cambridge, UK): Anti-TNF-α (1:1,000; cat. no. ab6671; rabbit anti-mouse), anti-TLR4 (1:2,000; cat. no. ab83444; rabbit anti-mouse), anti-TRAF6 (1:5,000; cat. no. ab33915; rabbit anti-mouse), anti-NF-κB (1:5,000; cat. no. ab32360; rabbit anti-mouse), anti-B-cell lymphoma 2 (Bcl-2)-associated X (Bax; 1:500; cat. no. ab32503; rabbit anti-mouse), anti-Bcl-2 (1:500; cat. no. ab59348; rabbit anti-mouse), anti-caspase-3 (1:1,000; cat. no. ab2302; rabbit anti-mouse), anti-GAPDH (1:10,000; cat. no. ab181602; rabbit anti-mouse). anti-IL-6 (1:1,000; cat. no. 12912; rabbit anti-mouse) and anti-IL-1β (1:1,000; cat. no. 12426; rabbit anti-mouse) were purchase from CST Biological Reagents Co., Ltd. (Shanghai, China). Atorvastatin was obtained from Pfizer, Inc. (New York, NY, USA).

Cell culture. Mice (C57BL6; 7-9 weeks old; 1:2 male:female) were purchased from Guangdong Medical Laboratory Animal Center. These mice were bred to produce the newborn mice. Parental and newborn mice were housed at 21±2˚C with 60% relative humidity, a 12 h light/dark cycle and free access to food and water. Six newborn mice (born <24 h; weight, 1-2 g) were euthanized via cervical dislocation and subsequently soaked and disinfected in 75% ethanol for 3-5 min at room temperature. Brains were removed via craniotomy. Brains were washed with cold D-Hanks solution (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) and the meninges and blood vessels were removed. Hippocampus tissues were sliced into 2 mm sections and washed in D-Hanks solution for 10 min at 37˚C. Cells were subsequently digested with 0.125% trypsin (Beyotime Institute of Biotechnology, Haimen, China) for 20 min at 37˚C. A total of 20 ml Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into cells via a pipette. The suspension was then collected and filtrated with an 80 mesh nylon filter (178 μm). The suspension was centrifuged at 800 x g for 15 min at 4˚C, the supernatant was discarded and 10 ml DMEM/F12 was added to the precipitate. Cell debris and residual tissues were sunk through centrifugation at 800 x g for 10 min at 4˚C and the cell suspension was collected. Cells were seeded into a pre-coated polylysine culture flask (75 cm^2, 250 ml) and incubated at 37˚C overnight in 5% CO_2. According to the metabolism of the cells being assessed, fluid was changed every 2-3 days, as described previously (28). After 2 weeks, the culture flask was agitated using a rotary shaker at speed of 300 rpm overnight at 37˚C. The supernatant cell suspension was removed and inoculated into a second culture flask. A total of 2 h following the renewal of culture medium, the adherent cells was BV-2 microglial cell line. The identification of microglia was determined by detecting the expression of GFAP by western blot analysis (data not shown) using an anti-GFAP antibody (cat. no. ab7260; 1:10,000).

Preparation of microglial-conditioned medium (MCM). BV-2 microglial cells were used to establish a model of activated microglia under OGD conditions as previously reported (29). Cells ($1x10^5$) were maintained in serum/glucose-free DMEM in an anaerobic environment for 1.5 h at 37˚C containing 5% CO_2 and 95% N_2. Cells were then transferred into DMEM supplemented with 1% B27, 2 mmol/l glutamine and 10% penicillin-streptomycin) and incubated in atmosphere containing 95% air and 5% CO_2 at 37˚C. Following a 48 h incubation, the MCM was harvested. Centrifugation (800 x g for 1 min at room temperature) was performed to purify the conditioned medium. The medium was subsequently diluted with serum-free DMEM to 1:1. Following this, the conditioned medium was used for the culture of OGD hippocampal neurons *in vitro*.

Primary culture of hippocampal neuronal cells. Primary hippocampal neuronal cell cultures were performed as described previously (30). The hippocampal formations of newborn mice (<24 h) were dissected and incubated with D-Hanks solution. The tissues were digested using 0.125% trypsin and grinded softly at 37˚C. Subsequently, the dissociated cells were scattered onto glass cover slips covered with poly-L-lysine (molecular weight, 20,000-50,000; 0.1 mg/ml; Sigma-Aldrich; Merck KGaA). Cells were then transferred into the MCM prepared in the aforementioned experiment to establish the OGD model. Cells were incubated under moist conditions at 37˚C and 5% CO_2 for 10-12 days. The medium was changed every 2-3 days.

Cell viability analysis. Cell viability was measured by performing an MTT assay. A total of ~5x10^4 cells/ml in the logarithmic phase were seeded into a 96-well plate and maintained for 12 h in an incubator (37˚C, 5% CO_2). Atorvastatin at five different concentrations (2.5, 5, 10, 20 and 50 μM) was added and cells were incubated at 37˚C and 5% CO_2 for 12, 24 and 48 h. Cell viability was determined by adding 10 μl MTT (5 mg/ml; Beyotime Institute of Biotechnology) solution and incubating at 37˚C for 6 h. Following centrifugation at 800 x g for 1 min at room temperature, the supernatant was discarded. Cells were treated with 100 μl dimethyl sulfoxide under low-speed oscillation for 10 min. A microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the absorbance at 490 nm. Growth values were calculated using optical density (OD) as follows: (OD treated cells/OD untreated cells) x100, as described in a previous study (31).

Cell apoptosis analysis. Flow cytometry (FCM) was utilized for the analysis of cell apoptosis. Cells were seeded at a density of 3x10^5 into a 6-well plate. Cells were treated with or without 10 μM atorvastatin at 37˚C for 24 h. Following OGD injury, cells were fixed in 70% ethanol at 4˚C for 2 h. Following this, cells for assessment were washed with PBS (containing KH_2PO_4, Na_2HPO_4, NaCl and KCl; Beijing Solarbio Science & Technology, Co., Ltd.) and resuspended in incubation buffer [10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid/NaOH (PH 7.4), 140 mmol/l NaCl, 5 mmol/l $CaCl_2$] at a density of 1x10^6 cells/ml. Cells were then maintained with annexin V fluorescein isothiocyanate (1 μg/ml) at 4˚C for 15 min and propidium iodide (1 μg/ml; Xilong Scientific Co., Ltd., Shenzhen, Luogang, China) at 4˚C for 5 min in the dark. A flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) was used for the analysis of cell apoptosis with CellQuest software version 5.1 (BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells in three groups: Control group; OGD only group; and OGD + 10 μM atorvastatin group. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 2 μg RNA was used for cDNA synthesis with a first strand cDNA kit (cat. no. 11483188001; Sigma-Aldrich; Merck KGaA) with the temperature protocol: 25˚C for 10 min, 42˚C for 50 min and 70˚C for 15 min. qPCR was performed using the SYBR Green Premix reagent (Takara Bio, Inc., Otsu, Japan) with an ABI 7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were utilized for qPCR: 10 min pretreatment at 94˚C, 55˚C for 40 sec and 70˚C for 40 sec (20 cycles), 94˚C for 30 sec, 65˚C for 40 sec and 70˚C for 40 sec (40 cycles), and a final extension at 70˚C for 10 min. It was then held at 4˚C. GAPDH was utilized as an internal control. The method of $2^{-\Delta\Delta Cq}$ was used for the normalization of expression to GAPDH (32). The primers used for the amplification in the present study are presented in Table I.

Western blot analysis. Cultured cells were lysed on ice with radioimmunoprecipitation buffer (pH 7.4; 0.1% SDS, 1 mM $MgCl_2$, 10 mM Tris-HCl, 1% Triton X-100). Debris was removed and the supernatant was collected following centrifugation at 800 x g for 1 min at room temperature. Protein concentration was confirmed using a bicinchoninic acid protein assay (Bio-Rad Laboratories, Inc.) following the manufacturer's protocol. An equal quantity (50 μg) of protein from each sample was separated using 5% SDS-PAGE gel and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) for 1.5 h. Following washing, non-specific sites were blocked by immersing membranes into 5% low fat dried milk at room temperature for 2 h. The membranes were subsequently maintained overnight with primary antibodies (anti-TNF-α, anti-TLR4, anti-TRAF6, anti-NF-κB, anti-Bax, anti-Bcl-2, anti-caspase-3, anti-IL-6, anti-IL-1β and anti-GAPDH) at 4˚C. Following washing with PBS, corresponding horseradish peroxidase-conjugated secondary antibodies (1:6,000; cat. no. ab6721; goat anti-rabbit IgG H&L) were incubated with the membranes at room temperature for 2 h. Signals were detected using an Enhanced Chemiluminescent™ detection kit (EMD Millipore). Band densities were quantified with Quantity One software version 4.6.9 (Bio-Rad Laboratories, Inc.).

ELISA. Quantikine ELISA kits for IL-6 (cat. no. SM6000B), IL-1β (cat. no. SMLB00C) and TNF-α (cat. no. SMTA00B) was purchased from R&D systems. All reagents mentioned in the following section were included in the ELISA kits. In brief, plates were removed from the sealed bag. A total of 100 μl standard products at different concentrations (1,000, 500, 250, 125, 62.5, 31.2, 15.6 pg/ml) were added into their corresponding wells. Wells were subsequently sealed with adhesive tape and incubated for 90 min at 37˚C. The biotinylated antibody working fluid was prepared in advance. All plates, excluding blank wells, were washed five times with PBS. A total of 100 μl biotinylated antibody working fluid was added to each well, sealed with adhesive tape and incubated for 60 min at 37˚C. The enzyme solution was prepared in advance for 30 min and then placed in the dark at room temperature. All plates, excluding blank wells, were washed five times. Enzyme solution (100 μl) was then added to each well, sealed with adhesive tape and maintained for 30 min at 37˚C. The plates were washed five times and the chromogenic substrate was added to each well and maintained in the dark for 10-15 min at 37˚C. A stop solution (100 μl) was then added to wells and agitated immediately for 10 min. The OD 450 value was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Table I. Sequences of primers utilized in reverse transcription quantitative polymerase chain reaction.

	Primer sequence	
Gene	Forward	Reverse
IL-6	CCAGTTGCCTTCTTGGGACT	TCTGACAGTGCATCATCGCT
IL-1β	ATCTCGCAGCAGCACATCAA	ATGGGAACGTCACACACCAG
TNF-α	CGGAAAGGACACCATGAGCA	GGGAGCCCATTTGGGAACTT
TLR4	CCGCTCTGGCATCATCTTCA	AGGTCCAAGTTGCCGTTTCT
TRAF6	CATGGACGCCAAACCAGAAC	TACACCTCTCCCACTGCTTG
NF-κB	CCTGCAACAGATGGGCTACA	TTCCTCCTTTGGGACGATGC
Bax	TCTCCGGCGAATTGGAGATG	CTCACGGAGGAAGTCCAGTG
Bcl-2	TGCGTGAAGGCTTGAGATGT	TCCCCCTTTCCTAGACCCAG
Caspase-3	TGGCTTGCCAGAAGATACCG	CCGTTGCCACCTTCCTGTTA
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC

IL, interleukin; TNF-α, tumor necrosis factor α; TRAF6, tumor necrosis factor receptor-associated factor 6; NF-κB, nuclear factor-κB; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

Statistical analysis. GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA,USA) was used to perform the statistical analysis. All data are presented as the mean ± standard error. At least three independent experiments were performed. The results of all assays were analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant result.

Results

Atorvastatin suppresses OGD BV-2 microglial cell viability. Conditions of OGD have been previously demonstrated to activate BV-2 microglia through the release of proinflammatory and neurotoxic factors, including IL-1, TNF-α and NO, which lead to the development of neuronal inflammatory injury (33,34). In addition, the anti-inflammatory effect of atorvastatin has been demonstrated to function in the protection of the CNS in AD (35,36). The present study assessed whether atorvastatin may affect the viability of OGD BV-2 microglia. Five different concentrations (2.5, 5, 10, 20 and 50 μM) of atorvastatin were applied to select the optimal concentration of atorvastatin for the subsequent experiments (data not shown) and 10 μM was selected. MTT results revealed that OGD led to significant decrease in BV-2 microglia viability ($P<0.05$ vs. control). Following atorvastatin treatment for 12, 24 and 48 h, it was revealed that the viability of OGD BV-2 microglia was increased compared with the OGD only group (Fig. 1A). These results indicated that atorvastatin rescued the viability of OGD-treated BV-2 microglia. Thus, it was hypothesized that atorvastatin may exert its effects through regulation of proinflammatory and neurotoxic factor release.

Atorvastatin reduces the expression of IL-6, IL-1β and TNF-α in OGD BV-2 microglia. The present study assessed the mechanism by which atorvastatin reduces the viability of OGD BV-2 microglia. The present study investigated whether the suppressive effect of atorvastatin on OGD BV-2 microglial cell viability may occur via effects on the release of proinflammatory and neurotoxic factors. Therefore, the expression of proinflammatory and neurotoxic factors in OGD BV-2 microglia treated with atorvastatin was assessed. ELISA results demonstrated that OGD significantly upregulated IL-6, IL-1β and TNF-α expression in BV-2 microglia ($P<0.01$ vs. control; Fig. 1B-D). However, IL-6, IL-1β and TNF-α expression in OGD BV-2 microglia was significantly decreased following atorvastatin treatment ($P<0.05$; Fig. 1B-D). In addition, RT-qPCR and western blotting indicated that atorvastatin treatment markedly reduced IL-6, IL-1β and TNF-α mRNA and protein expression in OGD BV-2 microglia ($P<0.01$; Fig. 2A and B). These results indicated that atorvastatin downregulated the expression of IL-6, IL-1β and TNF-α in OGD BV-2 microglia.

Atorvastatin downregulates the TLR4/TRAF6/NF-κB pathway in OGD BV-2 microglia. Furthermore, the expression of the TLR4/TRAF6/NF-κB pathway in OGD BV-2 microglia treated with atorvastatin was assessed using RT-qPCR and western blotting. The results revealed that OGD significantly increased the mRNA levels of TLR4, TRAF6 and NF-κB in BV-2 microglia ($P<0.01$; Fig. 2C), while the mRNA expression of these three genes was significantly reduced following treatment with atorvastatin in OGD BV-2 microglia ($P<0.05$; Fig. 2C). Furthermore, western blot analysis demonstrated that the OGD-induced overexpression of TLR4, TRAF6 and NF-κB proteins in OGD BV-2 microglia was significantly reduced following atorvastatin treatment, which was consistent with the results obtained from RT-qPCR ($P<0.05$; Fig. 2D). The multiple protein bands observed for TRAF6 and NF-κB presented Fig. 2D may have been caused by the specificity of the antibodies utilized in western blotting. Therefore, these results demonstrated that atorvastatin may suppress the TLR4/TRAF6/NF-κB pathway in OGD BV-2 microglia.

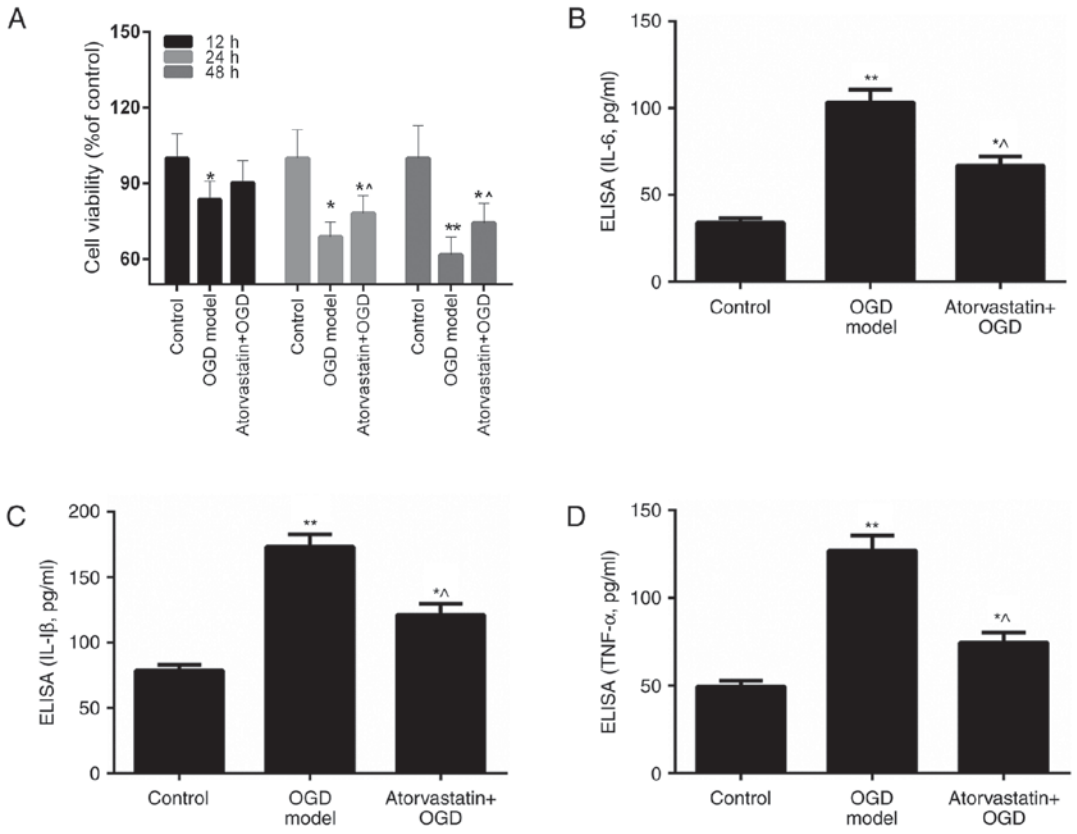

Figure 1. Atorvastatin affects the viability of OGD BV-2 microglia and the expression of inflammation factors. BV-2 microglial cells were placed under OGD conditions with or without treatment with 10 μM atorvastatin. (A) Cell viability was assessed using an MTT assay at 12, 24 and 48 h. ELISA was performed to determine the expression of (B) IL-6, (C) IL-1β and (D) TNF-α in BV-2 microglia following incubation with atorvastatin for 24 h. *P<0.05 and **P<0.01 vs. control; $^{\wedge}$P<0.05 vs. OGD model. OGD, oxygen-glucose deprivation; IL, interleukin; TNF-α, tumor necrosis factor-α.

Atorvastatin improves cell viability and inhibits apoptosis of hippocampal neuron. The viability and apoptosis of hippocampal neurons treated with OGD and atorvastatin were also assessed in the current study. Hippocampal neurons were cultured with MCM to create the OGD model. MTT results indicated that OGD evidently depressed the hippocampal neuron cell viability (P<0.05 vs. control), while additional atorvastatin treatment enhanced the viability of OGD hippocampal neurons (P<0.05 vs. OGD model; Fig. 3A). Furthermore, the anti-apoptosis ability of atorvastatin treatment on OGD hippocampal neurons was assessed. FCM data revealed that OGD led to a significant increase in the apoptosis of hippocampal neurons (P<0.001 vs. control; Fig. 3B and C). However, atorvastatin markedly inhibited the apoptosis rate of OGD hippocampal neurons (P<0.01; Fig. 3B and C). Furthermore, the expression of apoptosis-associated proteins in hippocampal neurons treated with OGD and atorvastatin was assessed. The results of RT-qPCR and western blotting demonstrated that the expression of Bax and caspase-3 in hippocampal neurons was significantly increased under OGD (P<0.001), while atorvastatin significantly downregulated Bax and caspase-3 expression in OGD hippocampal neurons (P<0.05; Fig. 4). However, Bcl-2 expression decreased under OGD (P<0.01) and was significantly enhanced following atorvastatin treatment (P<0.05; Fig. 4). These results verified the hypothesis that atorvastatin treatment may inhibit OGD hippocampal neuron apoptosis.

Discussion

AD is a chronic and polyfactoral neurodegenerative disease of the elderly. Previous studies have demonstrated that neuroinflammation induced by microglia is implicated in the pathogenesis of AD (37,38). As one of the most numerous immunocytes in the CNS, microglial cells have received attention in patients with AD due to their conspicuous response to AD pathophysiology (8,39). For example, it has been reported that the proinflammatory cytokines released from activated microglia, including IL-6, IL-1β and TNF-α, result in neuronal damage and loss (8,9). Lovastatin was previously reported to reduce the release of IL-6, IL-1β and TNF-α from rat primary microglia (40). However, the inhibitory effect of atorvastatin on the release of IL-6, IL-1β and TNF-α from activated microglia is yet to be elucidated. In the current study, mouse BV-2 microglia and hippocampal neurons induced by OGD were utilized to establish a model of AD neuronal inflammatory injury. The viability of BV-2 microglia treated with OGD and atorvastatin was assessed. The results indicated that atorvastatin treatment suppressed the viability of OGD BV-2 microglia. Due to these results, the present study further assessed the expression of proinflammatory cytokines in BV-2 microglia affected by OGD and treated with atorvastatin. The results demonstrated that atorvastatin significantly reduced the expression of IL-6, IL-1β and TNF-α in OGD BV-2 microglia. Therefore, it was concluded that atorvastatin may improve

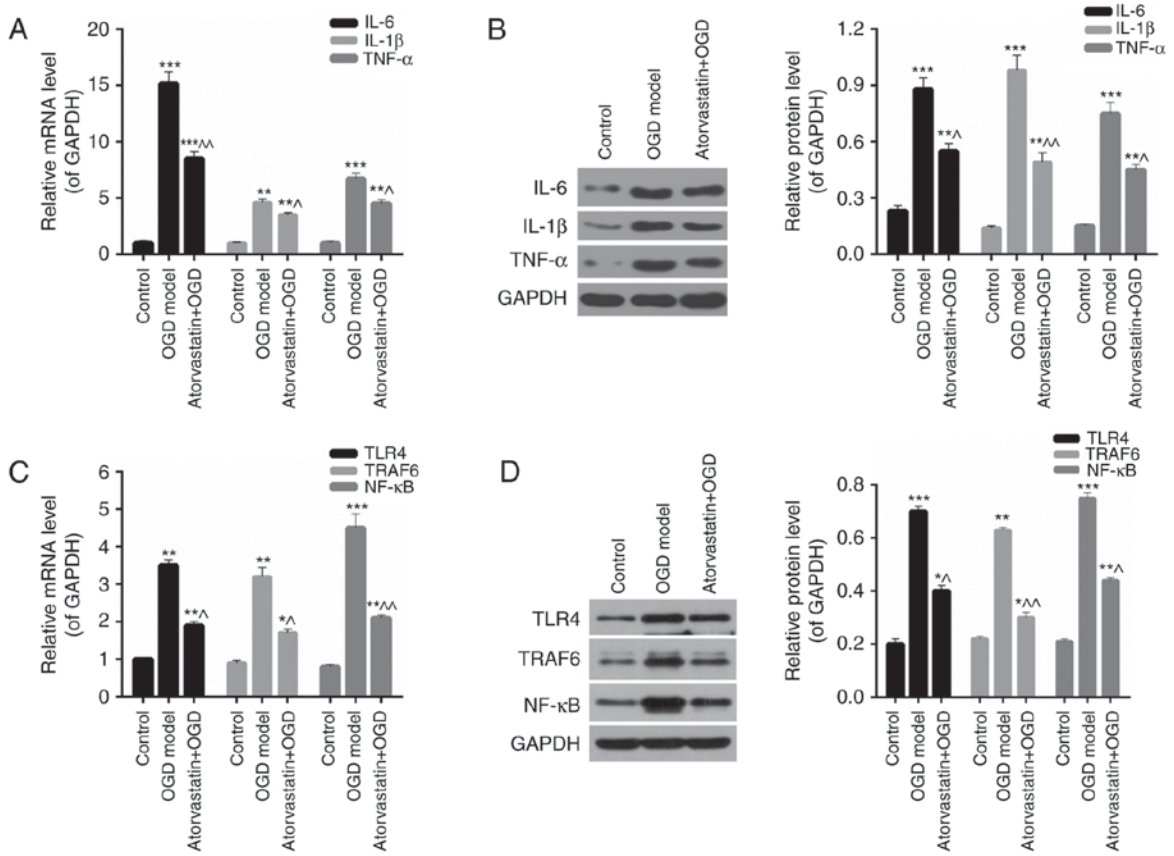

Figure 2. Atorvastatin downregulates the expression of inflammation factors and the TLR4/TRAF6/NF-κB pathway in OGD BV-2 microglia. BV-2 microglial cells were subjected to OGD conditions with or without treatment with 10 μM atorvastatin for 24 h. (A) RT-qPCR and (B) western blotting were performed to assess the expression of IL-6, IL-1β and TNF-α in BV-2 microglia. (C) RT-qPCR and (D) western blotting were performed to assess the expression of TLR4, TRAF6 and NF-κB in BV-2 microglia. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. OGD model. TLR4, toll-like receptor 4; TRAF6, tumor necrosis factor receptor-associated factor 6; NF-κB, nuclear factor-κB; OGD, oxygen-glucose deprivation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IL, interleukin; TNF-α, tumor necrosis factor-α.

the viability of OGD BV-2 microglia by downregulating the expression of IL-6, IL-1β and TNF-α.

TLR4, TRAF6 and NF-κB are crucial factors that participate in the regulation of inflammatory cytokine expression (41). A previous study demonstrated that suppression of the TLR4/NF-κB/signal transducer and activator of transcription signaling cascade mitigated microglial inflammation of the brain in AD (42). To the best of our knowledge, the present study was the first to assess the effect of atorvastatin on the TLR4/TRAF6/NF-κB pathway in neuronal inflammatory injury associated with AD. In the present study, the expression of TLR4, TRAF6 and NF-κB in BV-2 microglia with OGD and atorvastatin was assessed. The results revealed that the expression of TLR4, TRAF6 and NF-κB in BV-2 microglia was significantly upregulated by OGD, while atorvastatin treatment markedly reduced TLR4, TRAF6 and NF-κB expression in OGD BV-2 microglia. These results demonstrated that atorvastatin affected the TLR4/TRAF6/NF-κB pathway in OGD BV-2 microglia, indicating that atorvastatin may reduce the release of proinflammatory cytokines from OGD BV-2 microglia by suppressing the TLR4/TRAF6/NF-κB pathway. However, further investigation into the exact regulatory mechanisms between atorvastatin and the TLR4/TRAF6/NF-κB pathway are required.

It has been previously reported that cultured rat hippocampal neurons can be used to establish OGD neuronal injury models (43). Therefore, hippocampal neurons from mice were selected to further assess the protective effect of atorvastatin in OGD-induced neuronal inflammatory injury. MTT results revealed that atorvastatin increased the viability of OGD hippocampal neurons. According to the cell apoptosis analysis conducted in the present study, atorvastatin treatment significantly inhibited the apoptosis of OGD hippocampal neurons. Furthermore, the expression of apoptosis-associated proteins in hippocampal neurons treated with OGD and atorvastatin were also assessed. It was determined that OGD significantly increased the expression of Bax and caspase-3, and reduced the expression of Bcl-2, in hippocampal neurons. However, atorvastatin markedly downregulated the expression of Bax and caspase-3, and upregulated the expression of Bcl-2, in OGD hippocampal neurons. These results indicated that atorvastatin may inhibit OGD hippocampal neuron apoptosis by regulating the expression of Bax, Bcl-2 and caspase-3. In the current study, the results indicated that atorvastatin may protect mouse BV-2 microglia and hippocampal neurons from OGD-induced neuronal inflammatory injury by suppressing the TLR4/TRAF6/NF-κB pathway. However, the present study used *in vitro* model only. Therefore, to further elucidate the

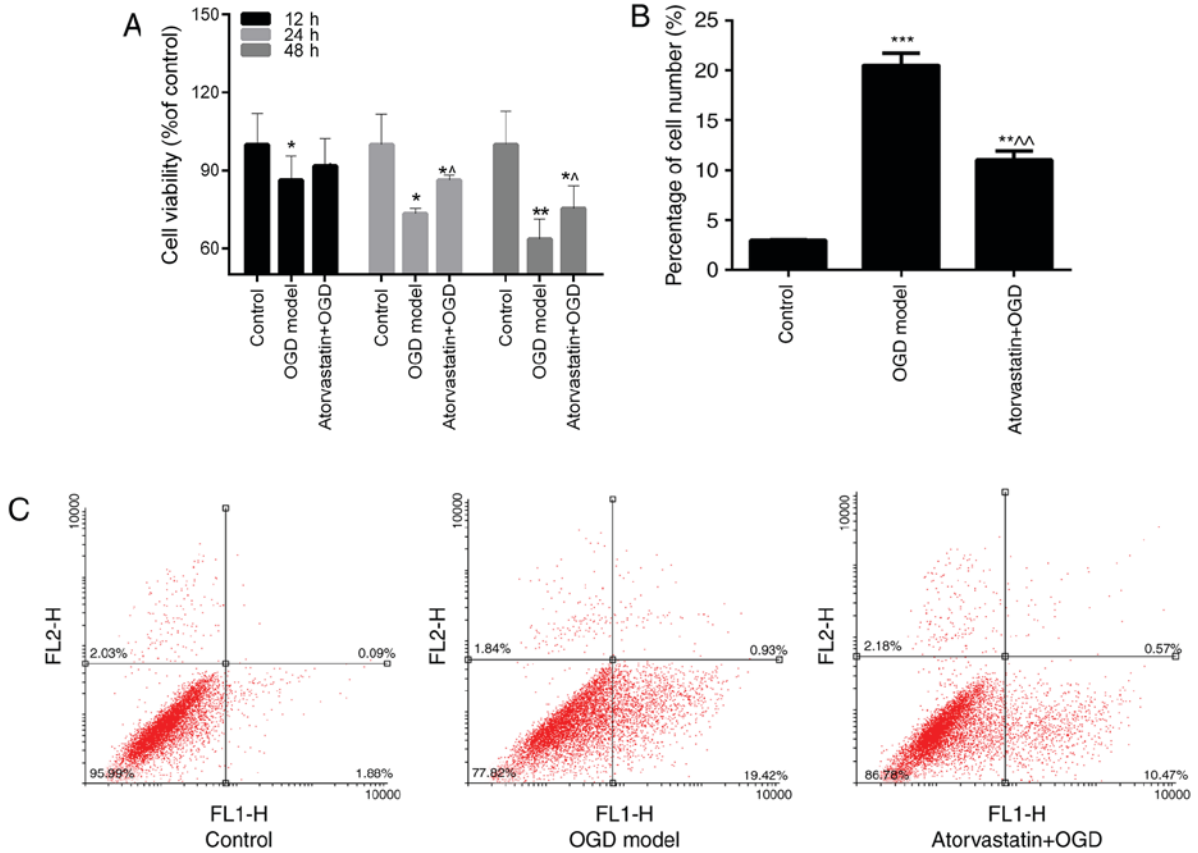

Figure 3. Atorvastatin suppresses the viability and apoptosis of OGD hippocampal neurons. Hippocampal neurons were treated with microglial-conditioned medium and 10 μM atorvastatin. (A) Cell viability was measured using an MTT assay and (B) Flow cytometry was performed to assess the apoptosis of hippocampal neurons following incubation with atorvastatin for 24 h. (C) Representative flow cytometry plots for control, OGD model and atorvastatin + OGD groups. The cells in upper and lower right quadrants were considered apoptotic cells. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. OGD model. OGD, oxygen-glucose deprivation.

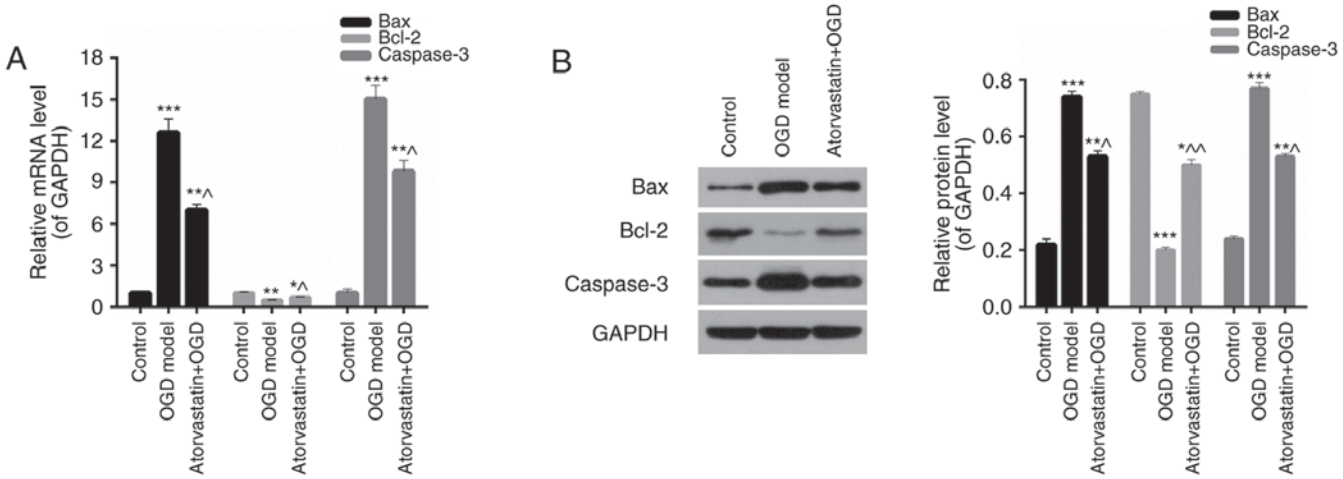

Figure 4. Atorvastatin regulates the expression of apoptosis-associated proteins in OGD hippocampal neurons. Hippocampal neurons were treated with microglial-conditioned medium and 10 μM atorvastatin. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blotting were performed to assess the expression of Bax, Bcl-2 and caspase-3 in hippocampal neurons. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\wedge}$P<0.05 and $^{\wedge\wedge}$P<0.01 vs. OGD model. OGD, oxygen-glucose deprivation; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

mechanism of neuronal inflammatory injury, an *in vivo* model or co-culture between microglia and neurons should be utilized.

In conclusion, the present study demonstrated that atorvastatin treatment may protect BV-2 microglia and hippocampal neurons from OGD-mediated neuronal inflammatory injury by suppressing the TLR4/TRAF6/NF-κB pathway. This conclusion may further support the utilization of atorvastatin in the treatment of AD.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

JH wrote the manuscript. JH, QY, YF and WS performed the experiments. JH and FG conceptualized the study design. WS and CZ analyzed the data. JH, QY and FG contributed to manuscript revisions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments in the present study were approved by the Institutional Review Board of Shaoxing Municipal Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Brown KL, Cosseau C, Gardy JL and Hancock RE: Complexities of targeting innate immunity to treat infection. Trends Immunol 28: 260-266, 2007.
2. Colton CA: Heterogeneity of microglial activation in the innate immune response in the brain. J Neuroimmune Pharmacol 4: 399-418, 2009.
3. Amantea D, Nappi G, Bernardi G, Bagetta G and Corasaniti MT: Post-ischemic brain damage: Pathophysiology and role of inflammatory mediators. Febs j 276: 13-26, 2009.
4. Lage JM: 100 Years of Alzheimer's disease (1906-2006). J Alzheimer Dis 9 (Suppl 3): S15-S26, 2006.
5. Aguzzi A, Barres BA and Bennett ML: Microglia: Scapegoat, saboteur, or something else? Science 339: 156-161, 2013.
6. Czirr E and Wyss-Coray T: The immunology of neurodegeneration. J Clin Invest 122: 1156-1163, 2012.
7. Lee CY and Landreth GE: The role of microglia in amyloid clearance from the AD brain. J Neural Transm (Vienna) 117: 949-960, 2010.
8. Perry VH, Nicoll JA and Holmes C: Microglia in neurodegenerative disease. Nat Rev Neurol 6: 193-201, 2010.
9. Swardfager W, Lanctot K, Rothenburg L, Wong A, Cappell J and Herrmann N: A meta-analysis of cytokines in Alzheimer's disease. Biol psychiatry 68: 930-941, 2010.
10. Eikelenboom P, Rozemuller AJ, Hoozemans JJ, Veerhuis R and van Gool WA: Neuroinflammation and Alzheimer disease: Clinical and therapeutic implications. Alzheimer Dis Assoc Disord 14 (Suppl 1): S54-S61, 2000.
11. Wesselingh SL, Takahashi K, Glass JD, McArthur JC, Griffin JW and Griffin DE: Cellular localization of tumor necrosis factor mRNA in neurological tissue from HIV-infected patients by combined reverse transcriptase/polymerase chain reaction in situ hybridization and immunohistochemistry. J Neuroimmunol 74: 1-8, 1997.
12. Matusevicius D, Navikas V, Söderström M, Xiao BG, Haglund M, Fredrikson S and Link H: Multiple sclerosis: The proinflammatory cytokines lymphotoxin-alpha and tumour necrosis factor-alpha are upregulated in cerebrospinal fluid mononuclear cells. J Neuroimmunol 66: 115-123, 1996.
13. Walter S, Letiembre M, Liu Y, Heine H, Penke B, Hao W, Bode B, Manietta N, Walter J, Schulz-Schuffer W and Fassbender K: Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. Cell Physiol Biochem 20: 947-956, 2007.
14. Kaczorowski DJ, Atsunori N, Mccurry KR and Billiar TR: Toll-like receptors and myocardial ischemia/reperfusion, inflammation, and injury. Curr Cardiol Rev 5: 196-202, 2009.
15. Stevens SL and Stenzel-Poore MP: Toll-like receptors and tolerance to ischaemic injury in the brain. Biochem Soc Trans 34: 1352-1355, 2006.
16. Gesuete R, Kohama SG and Stenzelpoore M: Toll-Like receptors and ischemic brain injury. J Neuropathol Exp Neurol 73: 378-386, 2014.
17. Hyakkoku K, Hamanaka J, Tsuruma K, Shimazawa M, Tanaka H, Uematsu S, Akira S, Inagaki N, Nagai H and Hara H: Toll-like receptor 4 (TLR4), but not TLR3 or TLR9, knock-out mice have neuroprotective effects against focal cerebral ischemia. Neuroscience 171: 258-267, 2010.
18. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K and Akira S: Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol 171: 4304-4310, 2003.
19. Kaisho T and Akira S: Toll-like receptor function and signaling. J Allergy Clin Immunol 117: 979-987; quiz 988, 2006.
20. Bi X, Yan B, Fang S, Yang Y, He J, Li XM and Kong J: Quetiapine regulates neurogenesis in ischemic mice by inhibiting NF-kappaB p65/p50 expression. Neurol Res 31: 159-166, 2009.
21. Valerio A, Dossena M, Bertolotti P, Boroni F, Sarnico I, Faraco G, Chiarugi A, Frontini A, Giordano A, Liou HC, *et al*: Leptin is induced in the ischemic cerebral cortex and exerts neuroprotection through NF-kappaB/c-Rel-dependent transcription. Stroke 40: 610-617, 2009.
22. Jin JJ, Kim HD, Maxwell JA, Li L and Fukuchi K: Toll-like receptor 4-dependent upregulation of cytokines in a transgenic mouse model of Alzheimer's disease. J Neuroinflammation 5: 23, 2008.
23. Endo A, Kuroda M and Tanzawa K: Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. FEBS Lett 72: 323-326, 1976.
24. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H and Zeiher AM: HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 108: 391-397, 2001.
25. Liao JK and Laufs U: Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol 45: 89-118, 2005.
26. Georgieva-Kotetarova MT and Kostadinova II: Effect of atorvastatin and rosuvastatin on learning and memory in rats with diazepam-induced amnesia. Folia Med (Plovdiv) 55: 58-65, 2013.
27. Zhang YY, Fan YC, Wang M, Wang D and Li XH: Atorvastatin attenuates the production of IL-1beta, IL-6, and TNF-alpha in the hippocampus of an amyloid beta1-42-induced rat model of Alzheimer's disease. Clin Interv Aging 8: 103-110, 2013.
28. Gordon R, Hogan CE, Neal ML, Anantharam V, Kanthasamy AG and Kanthasamy A: A simple magnetic separation method for high-yield isolation of pure primary microglia. J Neurosci Methods 194: 287-296, 2011.
29. Badiola N, Malagelada C, Llecha N, Hidalgo J, Comella JX, Sabriá J and Rodríguez-Alvarez J: Activation of caspase-8 by tumour necrosis factor receptor 1 is necessary for caspase-3 activation and apoptosis in oxygen-glucose deprived cultured cortical cells. Neurobiol Dis 35: 438-447, 2009.

30. Liu B, Zhang H, Xu C, Yang G, Tao J, Huang J, Wu J, Duan X, Cao Y and Dong J: Neuroprotective effects of icariin on corticosterone-induced apoptosis in primary cultured rat hippocampal neurons. Brain Res 1375: 59-67, 2011.
31. Li W, Zhai L, Wang H, Liu C, Zhang J, Chen W and Wei Q: Downregulation of LncRNA GAS5 causes trastuzumab resistance in breast cancer. Oncotarget 7: 27778-27786, 2016.
32. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
33. Hoehn BD, Palmer TD and Steinberg GK: Neurogenesis in rats after focal cerebral ischemia is enhanced by indomethacin. Stroke 36: 2718-2724, 2005.
34. Yrjänheikki J, Tikka T, Keinänen R, Goldsteins G, Chan PH and Koistinaho J: A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA 96: 13496-13500, 1999.
35. Clarke RM, O'Connell F, Lyons A and Lynch MA: The HMG-CoA reductase inhibitor, atorvastatin, attenuates the effects of acute administration of amyloid-beta1-42 in the rat hippocampus in vivo. Neuropharmacology 52: 136-145, 2007.
36. Cordle A and Landreth G: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors attenuate beta-amyloid-induced microglial inflammatory responses. J Neurosci 25: 299-307, 2005.
37. Alzheimer's Association: 2011 Alzheimer's disease facts and figures. Alzheimer Dement 7: 208-244, 2011.
38. Agostinho P, Cunha RA and Oliveira C: Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease. Curr Pharm Des 16: 2766-2778, 2010.
39. Streit WJ, Mrak RE and Griffin WS: Microglia and neuroinflammation: A pathological perspective. J Neuroinflammation 1: 14, 2004.
40. Pahan K, Sheikh FG, Namboodiri AM and Singh I: Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. J Clin Invest 100: 2671-2679, 1997.
41. Tang L, Zhou XD, Wang Q, Zhang L, Wang Y, Li XY and Huang DM: Expression of TRAF6 and pro-inflammatory cytokines through activation of TLR2, TLR4, NOD1, and NOD2 in human periodontal ligament fibroblasts. Arch Oral Biol 56: 1064-1072, 2011.
42. Capiralla H, Vingtdeux V, Zhao H, Sankowski R, Al-Abed Y, Davies P and Marambaud P: Resveratrol mitigates lipopolysaccharide- and Aβ-mediated microglial inflammation by inhibiting the TLR4/NF-κB/STAT signaling cascade. J Neurochem 120: 461-472, 2012.
43. Iijima T, Mishima T, Akagawa K and Iwao Y: Mitochondrial hyperpolarization after transient oxygen-glucose deprivation and subsequent apoptosis in cultured rat hippocampal neurons. Brain Res 993: 140-145, 2003.