Original Article

Atorvastatin prevents A β oligomer-induced neurotoxicity in cultured rat hippocampal neurons by inhibiting Tau cleavage

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Aim: The proteolytic cleavage of Tau is involved in A β -induced neuronal dysfunction and cell death. In this study, we investigated whether atorvastatin could prevent Tau cleavage and hence prevent A β ₁₋₄₂ oligomer (A β O)-induced neurotoxicity in cultured cortical neurons.

Methods: Cultured rat hippocampal neurons were incubated in the presence of A β Os (1.25 μ mol/L) with or without atorvastatin pretreatment. ATP content and LDH in the culture medium were measured to assess the neuronal viability. Caspase-3/7 and calpain protease activities were detected. The levels of phospho-Akt, phospho-Erk1/2, phospho-GSK3 β , p35 and Tau proteins were measured using Western blotting.

Results: Treatment of the neurons with A β O significantly decreased the neuronal viability, induced rapid activation of calpain and caspase-3/7 proteases, accompanied by Tau degradation and relatively stable fragments generated in the neurons. A β O also suppressed Akt and Erk1/2 kinase activity, while increased GSK3 β and Cdk5 activity in the neurons. Pretreatment with atorvastatin (0.5, 1, 2.5 μ mol/L) dose-dependently inhibited A β O-induced activation of calpain and caspase-3/7 proteases, and effectively diminished the generation of Tau fragments, attenuated synaptic damage and increased neuronal survival. Atorvastatin pretreatment also prevented A β O-induced decreases in Akt and Erk1/2 kinase activity and the increases in GSK3 β and Cdk5 kinase activity.

Conclusion: Atorvastatin prevents A β O-induced neurotoxicity in cultured rat hippocampal neurons by inhibiting calpain- and caspase-mediated Tau cleavage.

Keywords: Alzheimer's disease; atorvastatin; statins; hippocampus; neurotoxicity; amyloid- β peptide; Tau; calpain; caspase-3

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by abnormal accumulations of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) throughout cortical and limbic brain regions. Cognitive deficits in AD are believed to result from progressive synaptic dysfunction and neurodegeneration initiated by soluble amyloid β oligomers (A β Os) and further involving aggregates of hyperphosphorylated Tau^[1, 2]. A β Os, which are increasingly considered to act as proximal neurotoxins in AD, interact with glutamate receptors at the dendritic membrane, induce abnormal calcium influx and oxidative stress, block long-term potentiation (LTP), and facilitate long-term depression, ultimately leading to synapse failure^[3, 4]. It has recently been suggested that the deposition of A β might trigger a series of cel-

lular events that lead to posttranslational changes in Tau followed by neurite degeneration^[2, 5]. Furthermore, experiments in both cultured rodent hippocampal neurons and transgenic mice demonstrate that Tau is intrinsically involved in A β -mediated neuronal dysfunction and cell death^[6, 7]. Taken together, the data suggest that there is an intrinsic relationship between A β and Tau dysfunction. It has been shown that the deposition of A β induced the activation of calpain-1 and caspase-3 proteases^[2, 5, 8]. These proteases cleave Tau proteins at specific sites, generating toxic Tau fragments or enhancing the aggregation properties of Tau protein^[2, 5, 9]. The activation of these proteases, Tau cleavage, and the extent of neurodegeneration are both time- and dose-dependent^[2, 5, 9]. Inhibition of these proteases completely prevented the Tau proteolysis that leads to the generation of a 17-kDa fragment, significantly reducing A β -induced neuronal death^[2, 5]. Furthermore, hippocampal neurons transfected with 17-kDa Tau showed signs of degeneration, such as tortuous processes, varicosities along the neurites, and retraction of neuritic processes^[5]. Collectively,

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these data suggest that a reduction in factors that prevent the activation of calpain and caspase-3, and hence Tau cleavage, could have some bearing in the pathophysiology of AD.

Several prospective studies have indicated that statins could prevent the onset of AD^[10–12]. A recent meta-analysis by Wong *et al* also showed that statins have preventive effects on AD^[13]. Evidence from cell culture experiments and animal studies has suggested that statins have many pleiotropic effects, such as reducing A β production, suppressing inflammatory responses, protecting neurons from A β -induced neurotoxicity, apoptosis and oxidative stress, and promoting synaptogenesis^[14–17]. Recently, a transgenic mouse model of tauopathy showed a reduction in NFTs in response to statin treatment in both early and late stages of disease progression^[18]. It has been reported that statins reduce the number of phosphorylated Tau-positive neurites in aged amyloid precursor protein (APP) transgenic mice^[19]. Atorvastatin is a member of the statin family. Clarke *et al* demonstrated that rats treated with atorvastatin for 3 weeks were protected against a deficiency in LTP caused by the acute injection of A β _{1–42}^[20]. Our previous results revealed that atorvastatin prevented A β O-induced synaptotoxicity, which leads to memory dysfunction through a p38MAPK-dependent pathway^[17]. However, the mechanisms underlying the neuroprotective effects of statins have not been fully elucidated. In the present study, we analyzed whether atorvastatin exerts its neuroprotective effect against A β O-induced neurotoxicity by preventing Tau cleavage. Our results showed that atorvastatin blocked the activation of calpain and caspase-3 and hence decreased the generation of 17-kDa Tau fragments. Treatment with atorvastatin also decreased neurite degeneration in cultured hippocampal neurons treated with A β Os.

Materials and methods

Reagents

Atorvastatin was obtained from LKT Laboratories (St Paul, MN, USA). The calpain inhibitor Z-L-Abu-CONH-ethyl was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone was from Sigma-Aldrich (St Louis, MO, USA).

Preparation of A β Os

Human A β _{1–42} (product No. A9810) was purchased from Sigma-Aldrich (St Louis, MO, USA). A β Os were prepared according to our previously described method^[17].

Primary hippocampal neuron cultures

Hippocampal cultures were prepared from embryonic Sprague-Dawley rats as previously described^[21]. The hippocampi of E18/19 rat fetuses were collected in Hanks' solution without Ca²⁺ and Mg²⁺ (D-Hanks). The hippocampi were then mechanically fragmented, transferred to D-Hanks' solution containing 0.125% trypsin, and incubated for 15 min at 37°C. Following trypsinization, cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) and re-suspended in DMEM/F12 medium containing 10% heat-inactivated fetal bovine serum, 10% horse serum, glutamine (3 mg/mL), insu-

lin (0.25 mg/mL), penicillin (50 U/mL), and streptomycin (50 mg/mL). The cells were plated on poly-L-lysine-coated 16-mm-diameter coverslips (~150 cells/mm²) for immunocytochemistry assays, on 6-well culture plates (1×10⁶ cells/well) for Western blot analysis, or on 96-well dishes (1×10⁴ cells/well) for cell viability assays. Neurons were grown at 37°C in a humidified atmosphere of 5% CO₂/95% O₂. After 16 h, the medium was changed to neurobasal medium supplemented with glutamine (3 mg/mL) and B-27 (2%; Life Technologies, Gaithersburg, MD, USA). Subsequent half-medium changes were performed every 3–4 d for 14 d, at which time A β Os treatments were initiated.

Treatment of the cultures

Immediately after preparation of soluble A β Os, the solution was diluted to between 0.16 and 2.5 μ mol/L in neuronal culture medium. The hippocampal neurons, which had been cultured for 14 d, were incubated with A β Os for various time periods, ranging from 10 min to 48 h, with or without atorvastatin. In co-incubation experiments, atorvastatin or inhibitor was added to the neurons 1 h prior to incubation with A β Os. Inhibitors were added to the cell cultures 1 h prior to incubation with atorvastatin. The calpain inhibitor Z-L-Abu-CONH-ethyl was used at 1 μ mol/L. The caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone was used at 50 μ mol/L.

Cell viability assays

To examine the effect of atorvastatin treatment on cell viability, hippocampal neurons were plated directly in 96-well dishes (1×10⁴ cells/well) and incubated with A β Os for 48 h with or without atorvastatin (0.1, 0.5, 1, and 2.5 μ mol/L). In co-incubation experiments, atorvastatin was added to the neurons 1 h prior to incubation with A β Os. At the end of incubation, cell viability was determined by adding 100 μ L of CellTiter-Glo® luminescent reagent (Promega, Madison, WI, USA), which produces light in direct proportion to the amount of ATP and the number of viable cells present. The luminescence was measured using a BioTek Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA). The luminescent values were normalized between untreated control cells (100% viable) and a cell death control treatment of 200 μ mol/L staurosporine (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h (0% viable), which consistently gave luminescent readings only slightly above background (wells with culture medium but without cells). The CytoTox-ONE™ (Promega, Madison, WI, USA) assay measures lactate dehydrogenase (LDH) released into the culture medium through compromised cell membranes^[22]. One hundred microliters of medium was removed from the treated neurons and placed into 96-well dishes. LDH activity in the medium was measured according to the manufacturer's instructions, and fluorescent values were normalized between untreated controls (100% viable) and Triton X lysis controls to represent the maximum amount of LDH available for release (0% viable). Each experiment was performed in triplicate.

Immunofluorescence microscopy

After fixation with 4% paraformaldehyde for 30 min, neurons cultured on coverslips were permeabilized in PBS with 0.2% Triton X-100 for 5 min and incubated with 3% BSA in PBS for 30 min for the immunocytochemical analysis of microtubule-associated protein-2 (MAP-2, a dendritic marker), tubulin, Tau-5, PSD-95, and Bassoon. The cells were incubated with a mouse monoclonal anti-MAP-2 antibody (1:100; Novus Biologicals, CO, USA), mouse monoclonal anti-Tau (Tau-5, 1:100; Abcam Inc), rabbit polyclonal anti-Bassoon (1:100; Abcam Inc), or rabbit polyclonal anti-PSD-95 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody overnight at 4°C. After extensive washes with PBS, the cells were incubated with an anti-mouse or anti-rabbit secondary antibody conjugated with fluorescein (FITC, 1:1000; The Jackson Labs, West Grove, PA, USA) or rhodamine (TRITC, 1:1000; The Jackson Labs, West Grove, PA, USA).

Protease activity assays

Neurons cultured in 96-well dishes were directly measured for calpain and caspase-3/7 protease activities using substrates that release luminescent signals when cleaved. Calpain activity was measured using the Calpain-Glo assay (Promega, Madison, WI, USA). Luminescent measurements from A β O-treated wells (triplicate) were normalized to control, mock-treated wells (set to 1). Caspase-3/7 activity was measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) and analyzed in the same fashion as the calpain assay.

Western blot analysis

Western blot analysis was performed as previously described^[17]. Whole-cell lysates were prepared from hippocampal neurons cultured in 6-well dishes using radioimmune precipitation assay lysis buffer. After 30 min of lysis buffer treatment at 4°C, insoluble material was removed by centrifugation for 30 min at 12000 $\times$ g, and the protein concentration of the lysate was determined using a BCA assay. Equal amounts of protein were then separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with primary antibodies against the following proteins: mouse monoclonal anti-spectrin α II (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-vimentin (1:1000, Abcam, New Territories, HK, USA), mouse monoclonal anti-Tau (Tau-5, 1:500, Abcam, New Territories, HK, USA), rabbit polyclonal anti-p35 (C-19, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-phospho-GSK3 β (Ser9, 1:1000, Cell Signaling Technology, Beverly, MA, USA), rabbit monoclonal anti-phospho-Erk1/2 (Thr202/Tyr204, 1:1000, Cell Signaling Technology, Beverly, MA, USA), and rabbit monoclonal anti-phospho-Akt (Ser473, 1:1000, Cell Signaling Technology, Beverly, MA, USA). After being washed with PBS, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2000, Cell Signaling Technology, Beverly, MA, USA). Then, the membranes were washed and visualized using the ECL kit (Pierce, Rockford, IL, USA). The membranes were then re probed for β -actin immunore-

activity using a mouse anti- β -actin antibody (1:2000, Cell Signaling Technology). To determine the phosphorylation ratio of Akt, GSK3 β , and Erk1/2, the membranes were re probed with rabbit monoclonal anti-Akt (1:1000, Cell Signaling Technology, Beverly, MA, USA), rabbit monoclonal anti-GSK3 β (1:1000, Cell Signaling Technology, Beverly, MA, USA), or rabbit monoclonal anti-Erk1/2 (1:1000, Cell Signaling Technology). Staining intensity was quantified from 4 blots derived from 4 independent experimental trials. The density of each band was quantified using Image J software and normalized to total kinase or β -actin expression. The protein levels were expressed as a ratio of the band intensity for the protein of interest to that for total kinases or β -actin, which were used as loading controls.

Statistical analysis

The data are expressed as the mean $\pm$ SEM and were compared by one-way ANOVA followed by an LSD *post hoc* multiple-comparison test. $P < 0.05$ was considered statistically significant.

Results

Atorvastatin protected hippocampal neurons against soluble A β O-induced neurotoxicity

First, we determined the ability of different doses of soluble A β Os to induce neuronal death in primary rat hippocampal neurons using two independent assays. Our initial cell viability assay, measuring ATP content in neurons, revealed a dose-dependent effect, with soluble A β O treatment (0.625–2.5 μ mol/L) generating a significant decrease in cell viability over a 48 h exposure (Figure 1A). We used an independent cell viability assay that measures the release of lactate dehydrogenase into medium, reflecting a compromised membrane structure. The pattern of LDH release in this assay was similar to the loss of ATP in the cell viability measurements described above (Figure 1A). A concentration of 1.25 μ mol/L A β Os was chosen for all subsequent analyses because this concentration is comparable with many previous cell culture studies that assessed A β -induced cell death. When neuronal cell viability was measured over 48 h of exposure to 1.25 μ mol/L A β Os, pretreatment with atorvastatin produced significant protection (Figure 1B). We also examined the effect of atorvastatin on A β O-mediated neuronal cell death using the LDH assay, holding the A β O concentration at 1.25 μ mol/L. As shown in Figure 1B, atorvastatin provided significant, dose-dependent protection against A β -mediated cell damage. We next used protease inhibitors to determine whether the A β O-induced increase in protease activities contributed to A β -mediated neuronal cell damage. As shown in Figure 1B, both inhibitors provided considerable protection against neuronal death with the calpain inhibitor providing more protection than the caspase-3/7 inhibitor.

The experiments described above suggested that atorvastatin could block A β O-induced neurotoxicity. We next examined neuronal cell morphology 48 h after A β O treatment using an immunofluorescence microscope to image MAP-2,

Figure 1. Atorvastatin protected neuronal viability upon AβO treatment in cultured hippocampal neurons. Hippocampal neurons cultured for 8 d were incubated with the indicated concentrations of AβOs (A) for 48 h with or without the indicated concentrations of atorvastatin (B). In co-incubation experiments, atorvastatin was added to the neurons 1 h prior to incubation with AβOs. Cell viability was measured by ATP content and by LDH released into the medium. Data are expressed as the mean±SEM of 3 independent experiments. ^c*P*<0.01 compared with the control group; ^f*P*<0.01 compared with the AβO-treated group.

tubulin, and Tau. As shown in Figure 2, apparent signs of degeneration were detected 48 h after hippocampal neurons were treated with soluble AβOs for 48 h. The anti-tubulin images demonstrated that neurons displayed neuritis with varicosities as well as neuritis undergoing retraction. In contrast, no significant degenerative changes were detected in neurons treated with atorvastatin before treatment with AβOs (Figure 2). The anti-MAP2 images demonstrated that the dendrites of AβO-treated neurons were thinner and shorter than those of untreated neurons with a frequently fragmented or beaded appearance^[23]. However, neurons exposed to AβOs plus atorvastatin, a calpain inhibitor or a caspase-3/7 inhibitor for 48 h retained relatively normal dendritic morphology (Figure 2). The anti-Tau (Tau-5) images demonstrated that Tau was mainly located in neurites in control-treated neurons. However, treatment of neurons with AβOs (1.25 μmol/L,

48 h) induced a significant decrease in Tau signal in the distal regions of the neurites and a clear redistribution of Tau into the somata. In contrast, atorvastatin treatment blocked the AβO-induced decrease in Tau signal and Tau missorting (Figure 2). Similarly, both inhibitors prevented the AβO-induced decrease in Tau signal and Tau missorting (Figure 2).

Because synaptic damage likely plays a more important role than neuronal loss in the early stages of AD, we investigated whether atorvastatin could block AβO-induced synaptic damage. Two different synaptic markers were used to identify synapses: Bassoon, a large cytosolic scaffolding protein that is specifically concentrated at the presynaptic active zones of synapses^[24], and PSD-95, a postsynaptic scaffolding protein that plays a critical role in synaptic plasticity^[25]. Immunohistochemical detection of the Bassoon protein revealed that Bassoon exhibited a small punctate pattern on hippocampal neurons (Figure 3). Treatment of hippocampal neurons in culture with AβOs for 48 h induced a marked reduction in the density and signal intensity of Bassoon puncta. Pretreatment with atorvastatin (1 μmol/L) blocked the AβO-induced decrease in Bassoon immunoreactivity (Figure 3). To determine whether calpain and caspase are involved in the AβO-induced decrease in Bassoon expression, we observed the effect of both protease inhibitors on Bassoon immunoreactivity. After pretreatment for 1 h with a calpain or caspase inhibitor, we exposed neurons to AβOs for 48 h. Immunohistochemical detection demonstrated that both inhibitors protected against the AβO-induced decrease in Bassoon expression (Figure 3).

PSD-95 is predominantly localized at synapses. To assess whether atorvastatin prevented AβO-induced decreases in synaptic PSD-95, we treated hippocampal neurons (14 d) with soluble AβOs with or without atorvastatin for 48 h and immunostained them for PSD-95. Immunofluorescence images of PSD-95 demonstrated that treatment with AβOs decreased both the density and intensity of synaptic PSD-95-labeled puncta (Figure 3). Pretreatment with atorvastatin markedly attenuated the effect of AβOs on synaptic puncta density and intensity (Figure 3). In addition, both protease inhibitors prevented AβO-induced effects on the density and intensity of synaptic PSD-95-labeled puncta. These results suggest that calpain and caspase-3/7 contribute to AβO-induced neurotoxicity.

Atorvastatin prevented soluble AβO-induced activation of calpain and caspase-3/7 proteases

To further clarify the mechanism by which atorvastatin protects hippocampal neurons against AβO-induced neurotoxicity, we performed calpain and caspase-3/7 activity assays on hippocampal neurons as a function of time in the presence of AβOs using a Calpain-Glo^{TM} protease assay and a Caspase-Glo® 3/7 assay (Figure 4A and 4B). We observed rapid induction of calpain activity, reaching a maximum at 10 min (a 3-fold increase) and remaining elevated throughout the rest of the period analyzed (Figure 4A). Caspase-3/7 activity was induced at 10 min but was smaller in magnitude (~1.4 fold) and returned to control levels after 1 h of AβO treatment

Figure 2. Atorvastatin prevented A β O-induced neurotoxicity in cultured hippocampal neurons. Hippocampal neurons cultured for 14 d were treated with atorvastatin (1 μ mol/L) 1 h before incubation with soluble A β Os (1.25 μ mol/L) for 48 h. No signs of neurite degeneration were detected in untreated controls. In contrast, severe neurite degeneration was observed in cultures incubated with soluble A β Os. Atorvastatin significantly reduced the appearance of dystrophic neurites induced by A β Os. Insets are close-ups of the degeneration shown in the boxed regions. The redistribution of Tau into the somatodendritic compartment is indicated by arrowheads.

(Figure 4B). Pretreatment of neurons with atorvastatin (1 μ mol/L) diminished A β O-mediated calpain and caspase-3/7 activation (Figure 4A and 4B).

The inhibitory effects of atorvastatin on A β O-mediated activation of calpain and caspase-3/7 were verified using immunoblot analysis of known substrates for calpain and caspase-3/7 in the lysates. Cleavage of spectrin from its full-

length form (240 kDa) to a 150-kDa fragment is specific for calpain activity and is commonly used a surrogate marker of this protease's activity^[5]. A β O treatment led to a significant decrease in full-length spectrin (240-kDa) and a concomitant increase in the 150-kDa degradation fragment in cultured hippocampal neurons (Figure 4C). In contrast, preincubation with atorvastatin significantly attenuated A β O-induced

Figure 3. Atorvastatin attenuated A β O-induced synaptic PSD-95 and Bassoon downregulation in cultured hippocampal neurons. Primary rat hippocampal neurons cultured for 14 d were pretreated (1 h) with atorvastatin (1 μ mol/L) or vehicle before exposure to A β O (1.25 μ mol/L). After fixation, neurons were immunostained for PSD-95 or Bassoon. Higher magnification micrographs of the boxed regions are shown in the panel to the right.

calpain activation, as shown by decreased 150-kDa spectrin immunoreactivity and normal full-length spectrin levels (Figure 4C). Additionally, A β O treatment led to the fragmentation of vimentin (a known caspase-3/7 substrate), and this fragmentation was effectively attenuated by pretreatment with atorvastatin (Figure 4D).

Atorvastatin hindered A β O-induced Tau cleavage

Because we observed the inhibitory effect of atorvastatin on calpain and caspase protease activities in A β O-treated neurons, we next used immunoblotting analysis to assess whether atorvastatin prevented Tau cleavage in A β O-treated neurons. For these experiments, we harvested whole cell lysates from

Figure 4. Atorvastatin prevented AβO-induced activation of calpain and caspase-3/7 proteases. (A and B) Direct activity measurements were performed as described under “Materials and methods” as a function of time exposed to AβOs with or without atorvastatin pretreatment. Data were normalized to 1 for mock-treated controls. (C and D) An immunoblot analysis verifies protease activity with substrate cleavage. The calpain substrate spectrin and the caspase substrate vimentin demonstrate cleavage into smaller molecular weight fragments upon AβO treatment; cleavage of both is prevented by atorvastatin pretreatment. The bar chart shows the semiquantitative analysis of spectrin and vimentin. Data are expressed as the mean±SEM of 3 independent experiments. ^c*P*<0.01 compared with the control group. ^e*P*<0.05, ^f*P*<0.01 compared with the AβO-treated group.

neurons that had been treated in the absence or presence of AβOs, atorvastatin, or both. Blots of these samples were labeled with a phosphorylation-independent Tau antibody (Tau-5), which detects both full-length Tau (~48 and ~66 kDa) and the 17-kDa Tau fragment^[26]. As shown in Figure 5, AβO treatment for 24 h caused a reduction in full-length Tau immunoreactivity and a concomitant increase in 17-kDa Tau band density compared with untreated controls. In contrast, no reduction of full-length Tau was detected and the production of 17-kDa Tau was almost completely abrogated when atorvastatin (1 μmol/L and 2.5 μmol/L) was added to

the culture medium of hippocampal neurons 1 h before the addition of AβO (Figure 5). To obtain insights into the roles of calpain and caspase-3 in AβO-induced Tau cleavage leading to the generation of the 17-kDa fragment, we blocked the activation of these proteases using specific inhibitors. The caspase-3/7 inhibitor significantly reduced the generation of the 17-kDa Tau fragment in the AβO-treated neurons (Figure 5). In contrast, the calpain inhibitor completely prevented the generation of this Tau fragment in AβO-treated hippocampal neurons (Figure 5).

Figure 5. Atorvastatin protected against Tau degradation in hippocampal neurons. Hippocampal neurons cultured for 14 d were treated with atorvastatin (0.5, 1, or 2.5 $\mu\text{mol/L}$) or inhibitor 1 h before incubation with soluble A β O (1.25 $\mu\text{mol/L}$) for 24 h. The blot was labeled with Tau-5 antibody. The bar chart shows the semiquantitative analysis of the 17-kDa Tau fragment. Data are expressed as the mean $\pm$ SEM of 3 independent experiments. ^c $P<0.01$ compared with the control group; ^f $P<0.01$ compared with the A β O-treated group.

Atorvastatin inhibited A β O-induced increases in Tau-targeting kinase GSK3 β and Cdk5 activity

GSK3 β activity could play a central role in controlling apoptosis and in the development of Alzheimer's disease. Because GSK3 β activity is inhibited by phosphorylation on Ser-9, we assayed the effect of atorvastatin on A β O-induced changes in the level of Ser-9 phospho-GSK3 β . As shown in Figure 6A, A β O-treated hippocampal neurons demonstrated a remarkable reduction in the level of phospho-GSK3 β . After pretreatment for 1 h with atorvastatin, we exposed hippocampal neurons to A β O for 24 h. Atorvastatin treatment prevented the A β O-induced decrease in the level of Ser-9 phospho-GSK3 β in a dose-dependent manner.

We next investigated whether atorvastatin inhibited the

A β O-induced increase in Cdk5 activity, which is widely believed to regulate normal and pathological Tau activity^[27]. Because p25 is generated by proteolytic processing of p35^[28], we indirectly assessed Cdk5 activity by immunoblotting with an antibody that recognizes both p25 and p35 and determined the relative abundance of each. Again, we observed significant production of p25 after A β O treatment. Pretreatment of neurons with atorvastatin effectively diminished the A β O-mediated Cdk5 activity (Figure 6B).

Atorvastatin suppressed A β O-induced downregulation of phospho-Akt and phospho-Erk1/2 protein expression

GSK3 β activity has been shown to be regulated by Akt^[29]. More specifically, Akt can phosphorylate GSK3 β at serine 9, thereby suppressing GSK3 β kinase activity^[30]. Akt is activated by phosphorylation at serine 473, and treatment of neurons with A β O significantly reduces the levels of phospho-Akt (Figure 7A). Therefore, we tested the effect of atorvastatin on the A β O-induced reduction in phospho-Akt. Pretreatment of neurons with atorvastatin effectively prevented the A β O-induced decrease in phospho-Akt levels (Figure 7A). In addition, A β O-treated hippocampal neurons demonstrated a significant reduction in the levels of phosphorylated Erk1/2 (Figure 7B). Pretreatment of neurons with atorvastatin also inhibited the A β O-induced decrease in phospho-Erk1/2 (Figure 7B).

Discussion

A β and Tau are the two major aggregating proteins in AD. The toxic effects of A β are upstream of changes in Tau; however, Tau is considered necessary for the progression of neurodegeneration^[6, 7]. The aim of this study was to determine whether atorvastatin could prevent A β O-induced Tau cleavage and neurotoxicity in cultured hippocampal neurons. We chose rat hippocampal neurons as an experimental system because the hippocampus is affected early in AD. A β O were prepared from A β ₁₋₄₂. This preparation contains a mixture of A β O plus some monomers^[17]. We assessed the effects of atorvastatin on hippocampal neurons that were acutely exposed to A β O. These conditions are likely to be more relevant to early-stage AD, when hyperphosphorylated and fragmented Tau have not reached detrimental concentrations^[31]. The results described above indicate that atorvastatin blocked A β O-induced calpain and caspase-3 activation in cultured hippocampal neurons. Furthermore, atorvastatin prevented Tau cleavage, leading to the generation of toxic fragments. Through this mechanism, atorvastatin could ameliorate the synaptic impairment and neurite degeneration associated with the pathological cleavage of proteins. Our findings identify an alternative molecular mechanism by which atorvastatin could, at least to some extent, protect hippocampal neurons from A β O-induced neurotoxicity.

Several clinical studies have previously shown that treatment with statins reduced the risk of AD^[32, 33] and improved cognitive function and neuropathological changes in AD patients^[34, 35]. Recently, it has been reported that statins could

Figure 6. Atorvastatin inhibited the AβO-induced increases in GSK3β and Cdk5 activities. (A) Representative immunoblot comparing phospho-GSK3β to total GSK3β in hippocampal neurons. Group data showing the normalization of phospho-GSK3β to total GSK3β, determined in each group from 3 experiments. (B) Representative immunoblot comparing p35 and the production of its proteolytic fragment, p25 (an activator of Cdk5). Group data showing the normalization of p35 or p25 to β-actin, determined in each group from 3 experiments. Cultured hippocampal neurons at d 14 were pre-incubated with a vehicle solution (control, 0.1% DMSO) or atorvastatin (0.5, 1, or 2.5 μmol/L) for 1 h and then exposed to AβOs (1.25 μmol/L) for 24 h in the presence of vehicle or atorvastatin. Data are expressed as the mean±SEM of 3 independent experiments. ^c*P*<0.01 compared with the control group. ^f*P*<0.01 compared with the AβO-treated group.

Figure 7. Atorvastatin prevented AβO-induced downregulation of phospho-Akt and phospho-Erk1/2 expression. (A) Representative immunoblot comparing phospho-Akt to total Akt in hippocampal neurons after the treatments indicated. Group data showing the normalization of phospho-Akt to total Akt as determined in each group from 3 experiments. (B) Representative immunoblot comparing phospho-Erk1/2 in hippocampal neurons after the treatments indicated. Group data showing the normalization of phospho-Erk1/2 to total Erk1/2 as determined in each group from 3 experiments. Data are expressed as the mean±SEM of 3 independent experiments. ^c*P*<0.01 compared with the control group; ^e*P*<0.05, ^f*P*<0.01 compared with the AβO-treated group.

reduce the number of senile plaques and Tau-positive neurites in APP transgenic mice^[19]. Of particular interest, some studies have shown that statin treatment markedly reduced Tau levels

and NFTs in cellular and mouse models of tauopathy^[18, 36]. A clinical observation showed that simvastatin decreased the amount of phosphorylated Tau in the cerebrospinal fluid of AD patients^[37]. Statins were also reported to reduce NFTs more significantly than senile plaques in cognitively normal subjects^[35]. These observations suggest that statins have a direct effect on Tau pathology regardless of plasma cholesterol levels. In addition, atorvastatin suppressed Tau hyperphosphorylation induced by excess cholesterol in rat brains^[38]. In agreement with these findings, our present results showed that atorvastatin treatment could inhibit the A β O-induced activation of calpain and caspase-3 that leads to the generation of Tau-cleaved products with toxic functions in hippocampal neurons.

Direct evidence that Tau is involved in A β -induced neurotoxicity in central neurons has recently been obtained^[6]. This study showed that neurons expressing either mouse or human Tau proteins degenerated in the presence of A β , whereas Tau-depleted neurons showed no signs of degeneration in the presence of A β ^[6]. More recently, it has been suggested that proteolytic cleavage of Tau is also involved in the pathogenesis of AD. Caspase-3- and calpain-mediated Tau cleavage appeared to occur early in A β -induced neurotoxicity and to precede Tau hyperphosphorylation^[2, 5, 9]. Caspase-3-truncated Tau has been detected before the formation of NFTs and cell death^[39]. In addition, A β -induced calpain-mediated Tau cleavage that leads to the generation of the 17-kDa fragment has also been detected before enhanced Tau phosphorylation or neurite degeneration in cultured hippocampal neurons^[5]. Time-course experiments showed that the most rapid events upon treatment of hippocampal neurons with A β were induction of calpain and caspase-3/7 activity. Furthermore, the early protease activity affected Tau function, degraded full-length Tau and created low molecular weight Tau fragments^[2]. Indeed, calpain inhibition completely eliminated A β -mediated production of 24- and 17-kDa Tau fragments, and caspase inhibition reduced the production of the 17-kDa fragment. How cleaved Tau causes neuronal degeneration and/or neuronal death is not clear. One potential mechanism involves truncated Tau, which lacks the C-terminal 20 aa, assembles more rapidly into filaments than full-length Tau^[9]. The formation of Tau filaments could then interfere with different cellular processes. Another potential mechanism is that A β -mediated production of Tau fragments might promote Tau aggregation, which could be inherently toxic, as has been suggested in previous cell culture studies^[40]. In addition, cell death could be caused by the loss of normal Tau activity leading to misregulation of microtubule dynamics and microtubule function^[41, 42]. Another possibility is that Tau fragment-mediated neuronal death occurs through the activation of N-methyl-D-aspartate (NMDA) receptors^[43]. These results emphasize the potential of calpain and caspase-3 as therapeutic targets in neurodegenerative diseases. In our study, we found that the inhibitory effect of atorvastatin on the A β O-induced activation of calpain and caspase-3 was concentration-dependent. Our finding that atorvastatin was effective in preventing the A β O-induced

increase in calpain- and caspase-3-mediated Tau cleavage provides insight into the potential mechanism discussed above.

We also found that treatment of hippocampal neurons with A β Os induced clear redistribution of Tau into the cell bodies. These findings are similar to the results of a study by Zempel *et al*^[44]. In addition to early loss of synapses, the missorting of endogenous Tau from mainly axonal to somatodendritic compartments is among the earliest visible changes in AD. In dendritic regions from which Tau has been lost there is a depletion of spines, a local increase in Ca²⁺, and a breakdown of microtubules. Tau in these regions shows elevated phosphorylation at certain sites that are diagnostic of AD-Tau^[44]. Atorvastatin pretreatment prevented A β O-induced Tau missorting. This might be an alternative mechanism by which atorvastatin prevents A β O-induced synaptotoxicity. The mechanisms responsible for this effect require further examination.

In the present study, we also found that A β O treatment significantly reduced levels of phospho-Akt and phospho-GSK-3 β in hippocampal neurons, suggesting decreased activity of phospho-Akt and increased activity of GSK-3 β . These findings are similar to the results of a study by Reifert *et al*^[2]. It has been reported that within minutes of A β O treatment, a progression of calcium/calmodulin-dependent CaN activation is observed, first in dendritic spines and minutes to hours later in the cell body^[45]. Activated CaN dephosphorylates inhibitor-1, which leads to activation of protein phosphatase 1 (PP1)^[46]. PP1 activates GSK3 β by dephosphorylation of Ser-9^[47]. It has been postulated that GSK3 β activity might have a central role in the development of AD. GSK3 β activity was implicated in Tau phosphorylation, APP processing, A β production, and neurodegeneration^[48]. In addition, A β O-induced GSK3 β over-activity could lead to glutamate receptor internalization, spine retraction, LTP blockage, and LTD facilitation^[4, 49]. The blockade of either GSK3 β expression or activity decreased A β production and plaque accumulation^[50], improved performance in memory tests, preserved the dendritic structure, and reduced the Tau-dependent pathology in AD transgenic models^[51, 52]. In our study, we found that atorvastatin pretreatment prevented A β O-induced decreases in Ser-9 phosphorylation in hippocampal neurons, suggesting decreased activity of GSK3 β . Additionally, our previous study demonstrated that atorvastatin enhanced neuronal neurite outgrowth through up-regulation of the Akt/GSK3 β signaling pathway^[21].

Consistent with previous reports^[2, 53], we observed that phosphorylation of Erk1/2 was inhibited by A β Os in hippocampal neurons. However, our observation appears to contradict earlier reports^[54, 55] that suggested that synthetic A β causes activation of Erk1/2 in hippocampal slices or slice cultures. There are three key differences among these studies. First, we examined the direct effect of soluble A β Os on cultured hippocampal neurons, whereas Dineley *et al* documented the effect of A β ₁₋₄₂ on hippocampal slices. Second, our experiments used a much higher concentration of soluble A β Os than the nanomolar quantities of A β used by Dineley *et al*. Third, Dineley *et al* reported rapid activation of Erk within 5 min that was fol-

lowed by inactivation by 10 min.

In summary, our results indicated that atorvastatin prevented A β O-induced calpain- and caspase-3-mediated Tau cleavage. The decrease in the levels of Tau proteolytic forms was accompanied by increased cell survival in A β O-treated neurons. More studies are needed to fully elucidate the mechanisms by which atorvastatin prevents A β O-induced Tau cleavage and subsequent neuronal degeneration. Nevertheless, these observations provide further evidence of the beneficial effects of statins in the prevention of A β O-induced toxicity in central neurons.

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Author contribution

Ying JIN designed the research; Ling-ling ZHANG, Hai-juan SUI, and Zhou LIU performed the research; Hai-juan SUI analyzed the data; and Ying JIN wrote the paper.

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