Simvastatin ameliorates cognitive impairments via inhibition of oxidative stress-induced apoptosis of hippocampal cells through the ERK/AKT signaling pathway in a rat model of senile dementia

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Abstract. Senile dementia is a degenerative disease of the nervous system associated with cognitive impairments, memory disorders, executive dysfunctions, cognitive decline and dementia. Previous reports suggested that simvastatin presents ameliorative effects in the progression of senile dementia. However, the mechanism underlying simvastatin-mediated improvements of cognitive competence during the progression of senile dementia remains to be elucidated. In the present study, a potential mechanism underlying simvastatin activity in hippocampal cells, was investigated. Results of the present study demonstrated that simvastatin significantly improved cognitive impairments, memory competence, amyloid plaques, loss of neurons and synapses, neurofibrillary tangles and oxidative damage in experimental rats. Results of the present study demonstrated that administration of simvastatin regulates superoxide dismutase, reactive oxygen species, catalase and glutathione in oxidative stress processes in hippocampal cells. Apoptosis of hippocampal cells was suppressed by simvastatin treatment in rats with senile dementia. Notably, the administration of simvastatin inhibited activating transcription factor-6-mediated extracellular signal-regulated kinase/AKT serine/threonine kinase (ERK/AKT) signaling pathway in hippocampal cells. Taken together, the preclinical results of the present study indicate that simvastatin is efficient in preventing memory lapse and inhibiting apoptosis of hippocampal cells via the ERK/AKT signaling pathway, which may in the future improve cognitive decline and dementia in patients.

Introduction

Senile dementia is a progressive neurodegenerative syndrome characterized by cognitive function disorders and decline caused by degeneration and apoptosis of nerve cells in the brain and nervous system (1). Clinical manifestations of senile dementia include memory impairment, aphasia, damage of visual spatial skill, executive dysfunction and personality and behavioral dysfunctions (2,3). Neurodegenerative disorders in patients with senile dementia frequently result in an increased mortality rate in the elderly population (4,5). Systematic reviews and meta-analyses have investigated the association between statins and senile dementia. Statin drugs reduce the plasma concentration of cholesterol and have been reported to reduce the risk of senile dementia (6-8).

Previous reviews have discussed the effectiveness of statins in the prevention of dementia, but there is insufficient evidence to recommend statins for the treatment of Alzheimer's disease, a condition that leads to the development of numerous forms of dementia (9,10). Pathogenesis of senile dementia mainly targets the hippocampal area associated with memory and cognition, and leads to cognitive function disorders and impairments of cognition including, memory, language and attention (11,12). Elevated rates of apoptosis and oxidative stress in hippocampal cells are frequently observed in patients with senile dementia (13).

Apoptosis of hippocampal cells is a symptom of senile dementia (14,15). Petit et al (16) reported that the prevention of basal and induced neuronal apoptosis abrogates mutations associated with Parkinson disease. Previous reviews also indicated that apoptosis of hippocampal cells contributes to aggravation of senile dementia and leads to cognitive impairments, memory deterioration, formation of amyloid plaques, loss of neurons and synapses, and aggregation neurofibrillary tangles (17,18). These reports suggest that inhibition of apoptosis of hippocampal cells is beneficial for the suppression of cognitive impairments. A previous study indicated that memory dysfunction and failure of energy metabolism induced by oxidative stress are associated with the development of neurodegenerative disorders (19). Biomarkers of oxidative stress in patients with
vascular dementia have been observed in clinical patients with senile dementia (20). Furthermore, activation of the amyloidogenic pathway can cause oxidative stress, which leads to mitochondrial accumulation and apoptosis (21). Furthermore, protecting execution of hippocampal cells against apoptosis caused by oxidative stress in senile dementia disease was also clearly elaborated in previous study (22).

In the present study, the anti-apoptotic effects of simvastatin and potential molecular mechanism underlying the prevention of pathological processes of senile dementia in a rat model were investigated. A candidate signaling pathway triggered by simvastatin and leading to the improvement of cognitive impairment was evaluated in a rat model of senile dementia. The results of the present study indicated that simvastatin treatment significantly reduced amyloid plaques, loss of neurons and synapses and neurofibrillary tangles induced by oxidative stress, and apoptosis of hippocampal cells, through the extracellular signal-regulated kinase (ERK)/AKT serine/threonine kinase (AKT) signaling pathway in a rat model of senile dementia.

Materials and methods

Animal study. A total of 60 male Sprague Dawley rats (8 weeks old, 240-320 g) were purchased from the Chinese Academy of Sciences Institute of Biophysics (Beijing, China). All rats were intraperitoneally injected with scopolamine (1 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to establish a model of senile dementia (23). The injection was repeated five times once every three days and rats were divided into two groups (30 rats each). Rats with senile dementia were treated with a single daily intravenous 10 mg/kg/day simvastatin injection for 60 days or with an equivalent amount of PBS. All rats were housed at 24-26°C with a 12 h light/dark cycle and fed ad libitum.

Ethical approval. The present study complied with the Guide for the Care and Use of Laboratory Animals of China. All experimental surgeries and animal operations were performed in accordance with the Ethics of Animal Experiments Defense Research. During all surgical operations and euthanasia, efforts were made to minimize suffering. The present study was approved by the Ethics Committee of the Traditional Chinese Medicine Hospital of Weifang (Weifang, China).

Cell culture. Hippocampal cells were isolated from the CA1 region of rats with senile dementia as previously described (24). Hippocampal cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) and the culture medium was supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), penicillin and streptomycin (Sigma-Aldrich; Merck KGaA) and the culture medium was supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). Protein samples (20 µg) were concentrated in lysate buffer containing protease-inhibitor and were centrifuged at 8,000 x g at 4°C for 10 min. The supernatant was used for analyzing protein expression. Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, Inc.).

Western blotting. Hippocampal cells (1x10^6) were homogenized in lysate buffer containing protease-inhibitor and were centrifuged at 8,000 x g at 4°C for 10 min. The supernatant was used for analyzing protein expression. Protein concentration was measured by a BCA protein assay kit (Thermo Scientific Fisher Scientific, Inc.). Expression levels of superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and inducible nitric oxide synthase (iNOS) in hippocampal cells were measured by an RT-qPCR kit (AB4104C; Invitrogen; Thermo Fisher Scientific, Inc.) with β-actin as an endogenous control (25). The PCR cycling conditions were performed at 95°C for 30 sec and 45 cycles of 95°C for 5 sec, 56.5°C for 10 sec and 72°C for 10 sec. All primers (Table I) were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). Relative mRNA expression changes were calculated by the 2-ΔΔCq method (26). The results are expressed as a fold change compared with the β-actin control.

ATF-6 overexpression. Hippocampal cells (1x10^5) were cultured in a 6-well plate until 85% confluence; the media was then removed from the culture plate followed by 3 washes with PBS. Hippocampal cells were transfected by plentivirus-ATF-6 (ATFOP) using Lipofectamine 2000 (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols. After 48 h transfection, cells were used for further analysis.

Transfection of small interference RNA (Si-RNA). Hippocampal cells (4x10^4 cells/well) were seeded in a 6-well plate for 24 h at 37°C. The medium was removed and Opti-MEM (Invitrogen) added for 24 h at 37°C. The siRNA sequences corresponding to the gene were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences against ATF-6 (Si-ATF-6): 5'-GAAGGUGUUGA AUGUGGCAUCAA-3' or siRNA-vector (Control): 5'-UGC GUCUCAUGATGACAGTT-3'. After 48 h transfection, cells were used for further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from hippocampal cells using an RNAeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA). Expression levels of superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and inducible nitric oxide synthase (iNOS) in hippocampal cells were measured by an RT-qPCR kit (AB4104C; Invitrogen; Thermo Fisher Scientific, Inc.) with β-actin as an endogenous control (25). The PCR cycling conditions were performed at 95°C for 30 sec and 45 cycles of 95°C for 5 sec, 56.5°C for 10 sec and 72°C for 10 sec. All primers (Table I) were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). Relative mRNA expression changes were calculated by the 2-ΔΔCq method (26). The results are expressed as a fold change compared with the β-actin control.
Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>SOD</td>
<td>5′-TCTGGATGGTGTGCTTCTCTC-3′</td>
<td>5′-GCATGCTCCAAACATCGATC-3′</td>
</tr>
<tr>
<td>GSH</td>
<td>5′-GAAAGCCAGCTTTCTATTG-3′</td>
<td>5′-TTGGAACCGTGCTTAGTCTCA-3′</td>
</tr>
<tr>
<td>iNOS</td>
<td>5′-GACGAGACGGATAGCCAGG-3′</td>
<td>5′-CACATGCAAGGAAGGAACT-3′</td>
</tr>
<tr>
<td>CAT</td>
<td>5′-CGTGCTGAATAGGAAACAGA-3′</td>
<td>5′-AGTCAAGGTTGACGCTCTAGT-3′</td>
</tr>
<tr>
<td>Foxp2</td>
<td>5′-AGCAACCAGCTCTTCAGGTCC-3′</td>
<td>5′-ACGTGGTATTTGTGCTAGTACCCG-3′</td>
</tr>
<tr>
<td>SHIP</td>
<td>5′-CTCAAGATGCAATCCGGAAG-3′</td>
<td>5′-AAAGTTTTCTATAGGACCAAGC-3′</td>
</tr>
<tr>
<td>cREB</td>
<td>5′-GATACTCAGGCGAGATGCTACC-3′</td>
<td>5′-AGACCGAGGACACGACAAAGA-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-GTGGGCGCCAGGGCAACCA-3′</td>
<td>5′-CTCCTTATGTCACGCAGATT-3′</td>
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SOD, superoxide dismutase; GSH, glutathione; iNOS, inducible nitric oxide synthase; CAT, catalase; Foxp2, forkhead box protein P2; cREB, cAMP-response element binding protein.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. The TUNEL assay was used for the analysis of apoptosis of hippocampal neuron cells in experimental rats following simvastatin treatment (10 mg/kg/day; Sigma-Aldrich; Merck KGaA) or an equivalent dose of PBS. Procedures were performed as previously described (27). Briefly, cells (1x10^4) were cultured in a 6-well plate for 24 h at 37°C for 12 h. Then cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Subsequently, apoptosis of cells was stained with TUNEL reaction mixture (Sigma-Aldrich; Merck KGaA) at 37°C for 2 h. Cells were washed 3 times in TBST. TUNEL assays were conducted using a TUNEL fluorescence FITC kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Hippocampal neuronal cell images in 6 fields were captured using a Zeiss LSM 510 confocal microscope (Zeiss AG, Oberkochen, Germany) at a wavelength of 488 nm.

Cognitive tests. Cognitive competence of rats was determined by open field activity levels in a black Plexiglas box (60x60x25 cm) to analyze therapeutic effects of simvastatin. Rats were placed in the open black box for 10 min and their behavior was monitored and evaluated using an auto-tracking system (SmarTrack GPS Tracker; SmarTrack, Coalville, UK). The Morris water maze test was performed prior to and following the treatment with simvastatin to measure changes in cognitive competence. The Morris water maze experiment was performed in a circular stainless-steel tank (155 cm diameter, 60 cm depth) filled with water to a depth of 40 cm (27.0±1.0°C) that was made opaque by the addition of skim milk. Rats learned to find a hidden circular platform (10 cm diameter, 1.5 cm below the surface of the water) in a fixed area in one quadrant of the tank. A modified Rankin scoring (mRS) system (28) was used for assessing the therapeutic effects. Good outcomes and futile outcomes were defined as mRS scores ≤2 and 5-6, respectively. Rats tend to favor the closed arm when they feel strong anxiety (29).

Immunological staining. The effects of the simvastatin treatment on neuronal loss, amyloid plaques and neurofibrillary tangles were evaluated using immunohistochemical (IHC) staining for neuroprotection-associated proteins in hippocampi from experimental rats. Staining was performed on cerebral neurons of hippocampi of randomly selected animals from simvastatin- or PBS-treated groups. IHC procedures were previously reported in detail (30). Brains were frozen and coronal sections were cut in a cryostat following perfusion, fixation using 95% alcohol for 15 min at 37°C and cryoprotection. Free-floating sections (4 µm) were rinsed and placed in a solution containing anti-p75 nerve growth factor (NGF) receptor antibody (cat. no. ab8874; 1:500; Abcam) or anti-homocysteine (Hcy) antibody (cat. no. ab15154; 1:500; Abcam). Following rinsing, sections were incubated in the presence of a biotinylated horse anti-rabbit antibody (cat. no. a0545; 1:500; Chemicon; Merck KGaA) for NGF or Hcy staining for 2 h at 37°C. Sections were washed and observed using fluorescent video microscopy (BZ-9000; Keyence Corporation, Osaka, Japan).

Thionin staining. The hippocampal area from experimental rats was post-fixed in Böhm-Sprenger fixative (methanol, formalin, and acetic acid, in a 16:3:1 volume ratio) for 1 h at 37°C, hydrolyzed for 1 h in 5N hydrochloric acid, immersed in the thionin (Sigma-Aldrich; Merck KGaA) staining solution for 1 h, and rinsed thrice in a bisulphite rinse solution (0.5% sodium bisulphite (w/v) in 0.05N hydrochloric acid), each separated by water rinses. Images was observed by light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Statistical analysis. All data are expressed as the mean of triplicate experiments ± standard deviation and analyzed using Student’s t test or one-way analysis of variance followed by a Tukey honest significant difference post-hoc test. All data were analyzed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA), GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Simvastatin demonstrates beneficial effects on cognitive competence of hippocampal network in rats with senile dementia. In vivo efficacy of simvastatin on the treatment of senile dementia in rats was analyzed using cognitive
experiments. As presented in Fig. 1A, simvastatin treatment improved cognitive impairments determined by the escape latency during a 5-min study period. The memory competence, evaluated by the Rankin preclinical score of cognitive competences, was ameliorated by simvastatin treatment (Fig. 1B). Histological analysis of hippocampus demonstrated that amyloid plaques and loss of neurons were decreased in simvastatin-treated rats with senile dementia compared with the control (Fig. 1C and D). The results of the present study also demonstrated that the abundance of neurofibrillary tangles and anxiety was improved by simvastatin treatment in rats with senile dementia determined by Morris water maze (Fig. 1E and F). These results suggest that simvastatin treatment induces beneficial effects on cognitive competence of the hippocampal network.

Simvastatin inhibits apoptosis of hippocampal cells in the rat model of senile dementia. Apoptosis of hippocampal cells is associated with senile dementia. The efficacy of simvastatin in the prevention of apoptosis of hippocampal cells in the rat model of senile dementia was investigated. As determined by the TUNEL assay and presented in Fig. 2A, simvastatin (10 mg/kg/day) treatment inhibited apoptosis of hippocampal cells. The results of the present study demonstrated that simvastatin improved the viability of hippocampal cells compared with the control (Fig. 2B). Western blotting demonstrated that Bad and Bax were downregulated, while P53 and Bcl-2 were upregulated in hippocampal cells from the CA1 region in experimental rats compared with the control group (Fig. 2C and D). These results indicate that simvastatin potentially inhibits apoptosis of hippocampal cells in the rat model of senile dementia through the regulation of apoptosis-associated protein expression.

Simvastatin ameliorates oxidative stress of hippocampal cells in the rat model of senile dementia. Oxidative stress serves an essential role in the programmed hippocampal cell death. As presented in Fig. 3A and B, gene and protein expression levels of SOD and GSH were upregulated by simvastatin in hippocampal cells in the rat model of senile dementia. However, gene and protein expression levels of iNOS and CAT were downregulated in hippocampal cells in the rat model of senile dementia (Fig. 3C and D). These results indicate that simvastatin treatment ameliorates oxidative stress of hippocampal cells in the rat model of senile dementia.

Simvastatin improves neuroprotective protein expression in hippocampal cells in the rat model of senile dementia. The neuroprotective effect is an important aspect of anti-dementia drugs. Therefore, the present study analyzed changes in the expression of neuroprotective proteins in hippocampal cells in the rat model of senile dementia following treatment with simvastatin. As presented in Fig. 4A and B, simvastatin treatment increased gene and protein expression levels of Foxp2, SHIP and cREB in hippocampal cells. Immunohistochemistry demonstrated that simvastatin increased the expression of NGF and down-regulated Hcy expression levels in neurons (Fig. 4C). It was also observed that thionin staining of hippocampal area in simvastatin-treated rats demonstrated a marked difference in the dispersion of the pyramidal cell layer (Fig. 4D). These results indicate that simvastatin treatment can improve neuroprotective protein expression in hippocampal cells in the rat model of senile dementia.

Simvastatin regulates apoptosis of hippocampal cells through the ATF-6-mediated ERK/AKT signaling pathway. In order to analyze the potential mechanism underlying the activity of simvastatin in the progression of senile dementia, the ERK/AKT signaling pathway in hippocampal cells was investigated. As presented in Fig. 5A, expression levels of ATF-6, ERK and AKT were downregulated by simvastatin treatment in hippocampal cells compared with the control group. In vitro assays demonstrated that ATF-6 overexpression increased apoptosis in simvastatin-induced hippocampal
cells (Fig. 5B). Expression and phosphorylation levels of ERK and AKT were also increased by ATF-6 overexpression in hippocampal cells (Fig. 5C). However, ATF-6 knockdown enhanced apoptosis of hippocampal cells and downregulation of ERK1/2 and AKT expression levels in hippocampal cells (Fig. 5D and E). ATF-6 overexpression blocked simvastatin-regulated oxidative stress in hippocampal cells (Fig. 5F). These results suggest that simvastatin regulates apoptosis of hippocampal cells through ATF-6-mediated ERK/AKT signaling pathway.

Discussion

The majority of patients with senile dementia suffer from vascular dementia, which decreases the quality of their life (31). Senile dementia damages the nervous system and
impairs the cognitive ability in the elderly (32). Hippocampal cell apoptosis is highly correlated with hypoxia, ischemia and injury evidenced by changes in the infarct volume during chronic cerebral hypoperfusion (33). It has been demonstrated that simvastatin has a crucial role in apoptosis of hippocampal cells, memory recovery, cognitive rehabilitation and neuronal
survival (34). In addition, oxidative damage of brain regions may induce apoptosis of hippocampal cells, which could lead to cognitive dysfunction in patients with senile dementia (35). Furthermore, upregulation of the activity-dependent neuroprotective protein in the Tau mutation is regarded a novel marker for the onset of frontotemporal dementia (36). In the present study, the benefits of simvastatin for the treatment of senile dementia were further investigated and a potential mechanism of simvastatin-mediated anti-apoptosis in hippocampal cells was analyzed in a rat model of senile dementia.

Simvastatin is a statin that reduces hypercholesterolemia. Simvastatin has neuroprotective potential against 6-hydroxy-dopamine-induced Parkinson's disease-like symptoms (37). Neuronal marker recovery following simvastatin treatment of dementia in the rat brain was investigated in vivo by magnetic resonance (38). El-Dessouki et al (39) revealed that the neuroprotective effect of simvastatin is mediated via regulation of neurotransmitters and activity of acetylcholinesterase in L-methionine-induced vascular dementia. In addition, a previous study proposed a novel mechanism of simvastatin-mediated neuroprotection in neurodegenerative diseases through the modulation of seladin-1-associated metabolic regulation (40). A previous molecular analysis demonstrated that the neuroprotective effect of simvastatin is derived from the improvement of endoplasmic reticulum stress response in a rat model of global forebrain ischemia/reperfusion (41). In the present study, simvastatin improved cognitive impairments, memory competence, reduced the number of amyloid plaques and diminished the loss of neurons and synapses, neurofibrillary tangles and oxidative damage in experimental rats.

Apoptosis of hippocampal cells aggravates the degree of dementia and damages the cranial nerve system in patients with senile dementia (17). In the present study, simvastatin treatment suppressed apoptosis of hippocampal cells in CA1 regions and contributed to the improvement of cognitive competence. Previous research indicated that simvastatin exerts beneficial outcomes on patients with senile dementia due to the anti-neoplastic and anti-apoptotic effects in a number of cell types (42). In addition, attenuation of apoptosis, inflammation and oxidative stress in the blood and brain tissues is beneficial for the treatment of scopolamine-induced dementia in rats (23). A previous study suggested that ATF-6 is associated with apoptosis of dopaminergic neurons and accumulates in the core of Lewy bodies in Parkinson's disease (43). The results of the present study suggest that simvastatin treatment inhibits ATF-6-mediated ERK/AKT signaling pathway in hippocampal cells in CA1 regions in the rat model of senile dementia.

Expression of ERK is upregulated in hippocampal neurons of mice with vascular dementia (44). Hu et al (45) demonstrated that autophagy and AKT/cREB signaling are involved in the neuroprotective effects of nimodipine in a rat model of vascular dementia. Yao et al (46) demonstrated that ATF-6 mediates oxidized low-density lipoprotein-induced cholesterol accumulation and apoptosis in macrophages by upregulating DNA damage-inducible transcript 3 protein expression. In the present study, simvastatin treatment downregulated ATF-6, ERK and AKT expression in CA1 hippocampal cells in the rat model of senile dementia, which is consistent with a previous report (47). Endogenous expression of ATF-6 abolished the protective effects of simvastatin-inhibited neuronal damage in hippocampal cells in the CA1 region through the modulation of the activity of the ERK/AKT signal pathway.

In conclusion, the present study indicates that simvastatin may be an efficient agent for the treatment of senile dementia. Simvastatin treatment protects hippocampal neurons against apoptosis that further repairs the nervous system in the brain. The results of the present study indicate that simvastatin treatment suppresses apoptosis of hippocampal cells in the CA1 region through ATF-6-mediated ERK/AKT signaling pathway. These results suggest that simvastatin is a promising agent for the treatment of senile dementia.

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