Effect of atorvastatin on AGEs-induced injury of cerebral cortex via inhibiting NADPH oxidase -NF-κB pathway in ApoE−/− mice

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Abstract
Advanced glycation end products (AGEs) are a group of modified proteins and/or lipids with damaging potential. AGEs-RAGE pathway plays a critical role to induce neurodegenerative encephalopathy. Statins can reduce the expression of AGEs-induced AGEs receptor (RAGE) in the aorta. It is not clear whether statins have potential benefits on AGEs-induced cognitive impairment. In this study, the effects of atorvastatin (ATV) on inflammation and oxidation stress in the cerebral cortex were investigated, and the underlying mechanisms were explored. Apolipoprotein E (ApoE)−/− male mice were divided into four groups: control, AGEs, AGEs + ALT711 (Alagebrium chloride) and AGEs + ATV. β-amyloid (Aβ) formation in the cerebral cortex was assessed through Congo red staining and the functional state of neurons was evaluated by Nissl’s staining. Immunostaining was performed to assess the accumulation of AGEs in the cerebral cortex. The expressions of mRNA and protein of RAGE, Nuclear factor kappa B (NF-κB) p65 and Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) p47phox were detected by real-time polymerase chain reaction (PCR) and western blot. There were significant increases in AGEs deposit, Aβ formation, and the expressions of RAGE, NF-κB p65, and NADPH oxidase p47phox, and a decrease Nissl body in AGEs group compared with control group. ALT711 group recovered above change compared with AGEs group. Atorvastatin reduced Aβ formation and suppressed AGEs-induced expressions of NF-κB p65 and NADPH oxidase p47phox. Atorvastatin has little effects on AGEs deposit and RAGE expressions. Atorvastatin alleviates AGEs-induced neuronal impairment by alleviating inflammation and oxidative stress via inhibiting NADPH oxidase-NF-κB pathway.

Keywords Ages · RAGE · Atorvastatin · Central nervous system

Introduction
Advanced glycation end products (AGEs) are stable metabolic end products due to the slow non-enzymatic glycation of proteins (Maillard reaction), which occurs mostly during chronic diseases like diabetes [1]. AGEs formation is accelerated due to increased concentration of circulating glucose, AGEs precursors, and oxidative stress in the diabetic patients [2]. It was reported that AGEs could participate...
and accelerate the development and progression of diabetic complications, including neurodegenerative diseases [3]. Through direct cytotoxicity and interacting with receptors for AGEs (RAGE), AGEs could intensify the inflammatory response and oxidative stress within the brain [4]. AGEs binding to RAGE could transmit the signal from RAGE via redox-sensitive pathways to nuclear factor kappa B (NF-κB)-regulated cytokines [5–8]. AGEs-RAGE system triggers the generation of reactive oxygen species (ROS) by activating nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) [9], which activates NF-κB and induces subsequent transcription of related genes, including endothelin-1, tissue factor, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [10–12]. Additionally, activated NF-κB induces the expression of RAGE, which could interact with NF-κB to form a return circuit to amplify the neuronal deterioration process [10, 13]. Some findings indicated that RAGE-dependent signaling pathway could also facilitate β- and γ-secretase cleavage of amyloid precursor protein to generate β-amyloid (Aβ) through activating glycogen synthase kinase 3β and p38 mitogen-activated protein kinase [14]. Activated cytokines mediated neuronal death, synaptic impairment in the chronic inflammatory brain, and ROS in ageing could attack vital cell components and ultimately result in cell death.

Apolipoprotein E (ApoE), a main apolipoprotein in the brain, transports cholesterol between glial cells and neurons, lack of which leads to neurodegenerative dysfunction. Atorvastatin (ATV), 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, exerts cholesterol-lowering activity. Previous studies have suggested that statins have properties of anti-inflammatory and immunomodulatory functions and thus exhibit a neuroprotective effect [15, 16]. Our previous studies have demonstrated that atorvastatin could reduce the accumulation of AGEs and the AGEs-induced RAGE expression in the aorta independent of glucose levels [17, 18]. However, whether statins have neuroprotective effects on AGEs-related neurodegenerative encephalopathy is lacking. Based on the pleiotropic effects of statins, we hypothesized that atorvastatin could result in neuroprotective effects via ameliorating neuroinflammation and oxidative stress through down-regulating AGEs-induced activation of RAGE-NADPH oxidase-NF-κB.

Materials and methods

Animals

ApoE knockout (ApoE−/−) mice (age: 6 weeks; weight: 19 ± 1.7 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co.Ltd. (Beijing, China) and were allowed to adjust to their new environment for 2 weeks. The mice were housed at the animal facility of Shanghai East Hospital with free access to food and water and in a pathogen-free environment with a 12-h light/dark cycle. After the adjustment period, the animals were fed with a high-fat diet consisting of 42% fat, 19% crude protein, and 39% carbohydrate (Slaccas, Shanghai, China) at a controlled temperature of 22 ± 2 °C for 10 weeks. Those mice were divided into four groups:(1) AGEs group received AGEs-BSA 30 mg/kg/day via intraperitoneal injection (n = 10); (2) AGEs + ALT711 group received AGEs-BSA 30 mg/kg/day via intraperitoneal injection and ALT711, a type of crosslink breaker of AGEs (Ryan Chemical Co. Ltd., Suzhou, China) 1 mg/kg/day via intragastric administration (n = 10); (3) AGEs + ATV group received AGEs-BSA 30 mg/kg/day via intraperitoneal injection and atorvastatin (Jia Lin Pharmaceutical Co. Ltd., Beijing, China) 10 mg/kg/day via intragastric administration (n = 10); (4) Control animals received the same amount of saline solution (n = 10). All animal experimental procedures were in strict accordance with protocols approved by the Ethics Committee of Shanghai East Hospital, Tongji University and the experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. After 10 weeks high fat feeding, the fasting glucose, triglycerides, low-density lipoprotein, and high-density lipoprotein are comparable in the four groups of ApoE−/− mice (Supplementary materials Table 1S), as reported in our previous [19].

Preparation of AGEs-BSA

AGEs-BSA was prepared as described previously [20]. Briefly, D-glucose, bovine serum albumin (BSA), and penicillin–streptomycin were dissolved in 0.2 mmol/L PBS (pH 7.4) to the final concentrations of 0.5 mol/L, 50 g/L and 1 u/L respectively. The reaction mixture was incubated by protecting it from light for 24 h. They were filtered through a 0.22 μm filter and then incubated at 37 °C for 8 weeks. Removing the unconjugated glucose and to dialyze against sterilized PBS for 48 h. Fluorespectrophotometer with an excitation wave of 370 nm (the maximum absorption peak was measured at 440 nm), and SDS-PAGE (the molecular weight of the material was larger than BSA) that the mixture was glycated-BSA. The glycated-BSA was freeze-dried and stored at 4 °C.

Immunohistochemistry of AGEs

Immunostaining for AGEs was performed as described previously [21]. Briefly, brains were fixed with 4% paraformaldehyde, and 5 μm paraffin-embedded slide samples were routinely de-waxed, rehydrated, performed antigen retrieval with hot 10 mM sodium citrate buffer solution (pH 6.0) and rinsed in 3% H2O2 to block endogenous peroxidase. Then the
slides were incubated with a primary antibody against AGEs (1:500, Abcam) overnight at 4 °C and successively with a secondary antibody for 2 h at 37 °C. The signal amplification was performed using an avidin–biotin complex (ABC) HRP Kit (Beijing Zhongshan Jinqiao Biotechnology, Beijing China) and detected by DAB reagents. Nuclei were stained with hematoxylin, and dehydrated with ethanol series, and clearing with xylene. Images were obtained by a light microscope. Five sections of a cerebral cortex were selected, and the mean number of positive cells was recorded.

**Nissl’s staining and cell counting**

Nissl body is composed of a large number of rough endoplasmic reticulum and free ribosomes, which function is mainly to synthesize protein and used as a marker of the functional state of neurons [22]. Nissl’s staining was performed as described previously [23]. Briefly, the tissue sections (5 μm) were deparaffinized and subsequently rehydrated using different gradients of ethanol. Then the sections were stained in a 1% toluidine blue solution for 5–10 min, and differentiated in 75% ethanol for seconds, then rinsed quickly in distilled water. In addition, sections were counterstained with TO-PRO-3 Iodide (1:1000; Life Technologies) for the nonspecific nuclear staining of all cells. At last, sections were sealed with neutral gum. Five random fields of a cerebral cortex were selected and the mean number of positive cells was recorded.

**Congo red staining**

Congo Red histochemical stain may serve as a simple screening tool for investigating if the aggregates in mutant cells have misfolded β-pleated sheet secondary structures. Congo red staining was performed as described previously [24]. Briefly, brains were perfusion-fixed with 4% paraformaldehyde and paraffin-embedded. For Congo red staining, coronal brain sections were incubated for 5 min at room temperature in a solution containing 0.2% Congo red (Biopack, Argentina), 3% NaCl and 0.01% sodium hydroxide in 80% ethanol. After rinsing, sections were put on gelatin-coated slides, air-dried overnight, dehydrated using ethanol and cleared in xylene. At last, sections were sealed with neutral gum. Sections were visualized using optical microscopy for the detection of any orange amyloid plaques.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

RNA was extracted from cerebral cortex tissue, including RAGE, NF-κB p65 and NADPH oxidase p47phox, using TRIzol reagent (Sigma-Aldrich) according to the standard protocols [25]. Oligonucleotide primers were designed based on Genbank entries for rat RAGE, NF-κB p65, NADPH oxidase p47phox and β-actin. Primers sequence is presented in supplementary materials Table 2S. The following conditions were used for reverse transcription: 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min. Each 10 μl PCR contained 2.5 μl cDNA, 5 μl of 2 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 pmol of each primer. Quantitative PCR was performed in 96-well optical reaction plates on the ABI 7000 Real-Time PCR System with a procedure of denaturation step at 95 °C and annealing/extension step at 60 °C for 40 cycles, and melting curve analysis was used to verify the specificity and identity of target genes (Applied Biosystems). Relative gene expression was determined by the ΔΔCt method, where Ct meant threshold cycle. All experiments were performed in triplicate.

**Western blotting analysis**

Western blotting for RAGE, NF-κB p65 and NADPH oxidase p47phox in cerebral cortex was performed as standard protocols [25]. The brain tissue was taken out wholly, and a part of the cortex was soon separated. Then we homogenized the tissue in RIPA (Beyotime Biotechnology, China) followed with protease inhibitor cocktail (Roche, Basel, Switzerland). Next, we utilized the bicinechonic acid protein assay kit (Beyotime Biotechnology, China) to quantify the protein concentration. After that, we separated protein aliquots (30 μg) on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred them onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in TBST and incubated overnight at 4 °C with one of the following primary antibodies: Rabbit monoclonal RAGE (1:1000, Abcam), NF-κB p65 (1:1000, CST), NADPH oxidase p47phox (1:200, Santa) and β-actin (1:2000, Weiao). Then we washed membranes with TBST for 10 min × 3 and incubated with horseradish peroxidase-conjugated Goat anti-rabbit secondary antibodies at normal temperature for 1 h. Using the enhanced chemiluminescence reagent (Millipore, Bedford, MA, USA) and a gel imaging system (Bio-Rad, Hercules, CA, USA) to detect protein bands. The expression of target proteins was semi-qualified as the following formula: Relative coefficient = target protein concentration/β-actin concentration.

**Statistical analysis**

SPSS 20.0 (IBM, Chicago, IL, USA) was used for statistical analysis, and GraphPad Prism 6.04 software (San Diego, CA, USA) was used for figures. Variable distribution was detected by Shapiro–Wilk test, and P value less than 0.05 indicated a non-normal distribution. Quantitative analysis of AGES positive cells, Nissl’s staining,
Congo red staining, and western bolt was achieved by using Image J software v.1.37. Data were expressed as mean ± standard error (SEM). Since all data are in normal distribution, one-way analysis of variance was used to compare four groups, and post-hoc analysis of LSD was used to compare the two groups. $P < 0.05$ was considered as a statistically significant difference.

**Results**

**Deposition of AGEs in cerebral cortex**

As shown in Fig. 1, compared with control group, AGEs-positive cells were significantly increased in the cerebral cortex in AGEs group ($P < 0.001$). While compared with AGEs group, AGEs-positive cells were significantly decreased in the AGEs + ALT711 group ($P < 0.01$), but not in the AGEs + ATV group, suggesting that ALT711 could alleviate AGEs deposit in the cerebral cortex but not ATV.
The functional state of neurons

In control group, neurons exhibited a large amount of densely stained toluidine blue granules in the cytoplasm, and no histomorphological change was observed (Fig. 2). However, the Nissl bodies dramatically decreased or even disappeared, and the histomorphological arrangement was disordered in the AGEs group. Under treatment of atorvastatin or ALT711, there was a significant increase in Nissl bodies and improvement in disordered arrangement compared with AGEs group. These results suggested that AGEs could promote the injury of neurons in ApoE−/− mice, while both ATV and ALT711 could alleviate AGEs-induced injury of neurons.

Aβ plaque formation in cerebral cortex

In order to assess the Aβ plaque formation, Congo red staining was used. The Congo stained amyloid plaque numbers in the cortex remarkably increased in the AGEs group compared with control group. While in the atorvastatin group and ALT711 group, Congo red-stained plaques were obviously decreased compared with AGEs group (Fig. 3). These results indicated that ATV and ALT711 could efficiently inhibit AGEs-induced Aβ formation in cerebral cortex.

Expression of RAGE in cerebral cortex

There were significant increases in the expression of RAGE mRNA and protein in AGEs group compared with control
group. Compared with AGEs group, there were significant decreases in expression of RAGE mRNA and protein in group ALT711, but not in AGEs + ATV group (Figs. 4, 5). The results indicated that atorvastatin had little effect on the AGEs-induced expression of RAGE in the cerebral cortex.

Expression of NADPH oxidase p47phox and NF-κB p65 in cerebral cortex

There were significant increases in the expression of NADPH oxidase p47phox and NF-κB p65 mRNA and protein in both AGEs + ATV and AGEs + ALT711 groups (Figs. 4, 5). These results indicated that ATV could inhibit the transcription and expression of NADPH oxidase p47phox and NF-κB p65 in cerebral cortex.

Discussion

AGEs cause cognitive dysfunction through the AGEs-RAGE pathway or direct toxic effects, such as the release of reactive oxygen species and reduction of glucose consumption, ATP production and mitochondrial activity in neurons [26]. The RAGE ligands, including AGEs, high-mobility group box-1 protein, Aβ oligomers and S100 calgranulins, are secreted by microglia and immune cells [27]. Increasing levels of RAGE have been suggested as
a cause for amyloid aggregation in ageing and Alzheimer’s disease (AD) brains [28]. Excessive accumulation of AGEs and subsequent activation of RAGE are thought to be closely associated with either ageing or the development of diabetic complications [29]. RAGE transports circulating Aβ through the blood–brain barrier into brain parenchyma and interactions between RAGE and Aβ cause inflammatory responses and oxidative stress, and thus, reduce cerebral blood flow [30]. Glycated Aβ is hypothesized as a more suitable ligand for RAGE, and high levels of AGEs are associated with poorer learning in AD mice [29, 31]. Compared with the control group, the serum AGEs in the AGEs treatment group were significantly increased in another article of us [19]. A noticeable increase in RAGE levels and oxidative damage is observed in the brain of AD subjects, suggesting a vital role of ROS in the development AD [32, 33]. Activated microglia plays multiple roles in the pathology, which contributes to inflammation-related neuronal cell death in animal models of AD [27, 34]. NF-κB plays an important role in transducing inflammatory and pro-apoptotic signals, and RAGE-dependent activation of NF-κB leads to the up-regulation of RAGE itself [35, 36]. Additionally, activated microglia secretes large amounts of inflammatory mediators through NF-κB pathways, such as iNOS, TNF-α, and IL-6, which are generally considered prominent factors leading to neuroinflammation and neurotoxicity in neurodegenerative diseases [37]. This study showed that there was an obvious deposition of AGEs in the cerebral cortex, which induced neuronal damage with increased expression of RAGE, NADPH oxidase and NF-κB, suggesting that AGEs could activate RAGE-NADPH oxidase-NF-κB pathway to injury the cerebral cortex of mice.

Neuroprotective mechanisms of statins are a debating topic in the current medical literature. The pleiotropic effects of statins independent of cholesterol-lowering actions have been investigated, including anti-inflammatory, anti-oxidative stress, protection of the neurovascular unit, and facilitating exogenous Aβ degradation [38, 39]. Atorvastatin can down-regulate the RAGE gene directly in the aorta [17]. The over-expression of RAGE and NF-κB induced by ischemia after stroke was significantly attenuated by Atorvastatin [15]. But there was no statistically significant difference in decreasing expression of RAGE in our experiment, it suggests atorvastatin might be effective in decreasing RAGE in the cerebral cortex slightly. One study found that high-dose atorvastatin could significantly decrease TLR4, TRAF6 and NF-κB, reduce deposited Aβ inducing gliosis and simultaneously ameliorate impairments of spatial learning ability and memory in Aβ-injected rat [40]. Statin withdrawal increased NADPH oxidase, an important cellular source of superoxide in the cerebral cortex [41]. In our research, Atorvastatin could be significantly down-regulate NF-κB and NADPH oxidase, leading to decreased nerve injury and deposited of Aβ. These results provide evidence for the potent anti-inflammatory and antioxidant activity of atorvastatin. Therefore, atorvastatin could improve cognition function probably related to its effect of decreasing the activation of AGEs-RAGE system, which correlates with block of NADPH oxidase and NF-κB activation. At last, we suppose the proper modulation of RAGE-mediated signaling pathways might
be one of the effective therapeutic targets of atorvastatin for AGEs-induced neurodegenerative encephalopathy.

Limitations should be noted within the current study. The concentrations of serum cholesterol esters have not been measured, and it may have a certain effect on the results of our study. We did not do Morris water maze to evaluate brain nerve function of mice. Additionally, though we found that atorvastatin could down-regulated the expression of NF-κB and NADPH oxidase, the first step and the key part were unclear. Moreover, all of these signal pathways were reciprocal causation. Therefore, further researches are still needed to confirm the specific protective mechanism of statins.

**Conclusion**

Above all, our study demonstrated that atorvastatin could attenuate AGEs-induced encephalopathy, which AGEs-induced activation of RAGE-NADPH oxidase-NF-κB, via alleviating inflammation and oxidative stress through inhibiting NADPH oxidase-NF-κB pathway.

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**Author contributions** ZL participated in the design of the study, carried out the immunohistochemistry, performed the statistical analysis and drafted the manuscript. PY carried out the animal experiment, RT-PCR and Western blot. BF conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Data availability** The datasets used and analyzed during the current study are included in this published article.
Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval and consent to participate This study was conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health and with the approval by the Ethical Committee on Animal Research at Shanghai East Hospital of Tongji University (Shanghai, China).

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