Biphasic effects of statins on neuron cell functions under oxygen–glucose deprivation and normal culturing conditions via different mechanisms

Hui Wang | Yun Chen | Ping Li | Yan Chen | Danfang Yu | Qian Tan | Xiaoli Liu | Zhenli Guo

Abstract
While there is a growing interest in the use of statins, HMG-CoA reductase inhibitors, to treat neurodegenerative diseases, statins are associated with conflicting effects within the central nervous system (CNS) without clear evidence of the underlying mechanisms. This study systematically investigated effects of four statins (atorvastatin, pitavastatin, cerivastatin, and lovastatin) on neuronal cells under pathological condition using an in vitro model depicting ischemic injury, as well as tested under physiological condition. All four statins at micromolar concentrations display toxic effects on neuron cells under physiological condition. Atorvastatin and cerivastatin but not pitavastatin or lovastatin at nanomolar concentrations display protective effects on neuron cells under ischemic injury condition, via decreased ischemic injury-induced oxidative stress, oxidative damage, and inflammation. Mechanistically, atorvastatin, pitavastatin, and lovastatin induces neuron cell apoptosis via prenylation-independent manner. Other mechanisms are involved in the pro-apoptotic effect of cerivastatin. Prenylation is not involved in the protective effects of statins under ischemic injury condition. Our work provides better understanding on the multiple differential effects of statins on neuron cells under physiological condition and ischemic injury, and elucidate their underlying mechanisms, which may be of relevance to the influence of statins in CNS.

Keywords
neuron cells, OGD, oxidative stress, prenylation, statins

1 | INTRODUCTION

Ischemic stroke, a major cause of mortality and the main cause of long-term disability, is closely associated with cerebral ischemic/reperfusion injury. Thrombectomy and recombinant tissue plasminogen activator intravenous administration are standard of care treatments but not suitable for majority of patients due to the limited time window of therapeutic efficacy and intracranial hemorrhagic transformation. The pathophysiological processes of ischemic stroke are complex and include oxidative stress, inflammatory...
response, amino acid toxicity, and neuronal death.\(^2,3\) The aim of current work is to identify compounds that can alleviate neuron cell death induced by ischemic/reperfusion injury.

Statins are potent inhibitors of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase and a leading therapeutic class of lipid lowering drugs. As a result, statins are prescribed extensively for primary and secondary prevention of cardiovascular disease.\(^4\) Apart from cholesterol synthesis, HMG-CoA reductase plays an essential role in synthesis of mevalonate which is a critical enzyme of post-translational modification prenylation.\(^5\) The pleiotropic effects of statins have been extended to neurodegenerative diseases for its possible neuroprotective function.\(^6–8\) One study shows that statins may be effective in treating traumatic brain injury.\(^9\) However, toxic effects of statins on neuron cells are also observed and concerns over the neurological effects of statins have emerged.\(^10–12\) The effects of statins on neuron cells seem to be contradictory and underlying mechanisms of their differential actions remain largely unknown. For instance, lipophilic statins including atorvastatin, lovastatin, cerivastatin, and pitavastatin have been shown to cross readily into the central nervous system in animals. In contrast, hydrophilic statins, such as rosuvastatin and pravastatin, may not cross the blood–brain barrier.\(^13\)

In this work, we systematically investigated effects of four lipophilic statins (atorvastatin, pitavastatin, cerivastatin, and lovastatin) on neuron cell survival under ischemic injury and normal culturing conditions. We further analyzed the underlying mechanisms of statins focusing on inflammation, oxidative stress, and prenylation. Our work demonstrates that atorvastatin and pitavastatin but not cerivastatin and lovastatin at lower concentrations alleviate neuron cell death induced by oxygen–glucose deprivation (OGD). Interestingly, atorvastatin and pitavastatin at higher concentrations increase neuron cell death. We finally demonstrate that the biphasic effects of statins on neuron cell survival under normal culturing and OGD conditions are via different mechanisms.

\section{METHODS}

\subsection{Reagents, primary neuron cells, and neuron cell lines}

Atorvastatin, lovastatin, and pitavastatin were obtained from Selleckchem Inc. Cerivastatin was obtained from Sigma. All statins were >98\% pure based on HPLC data and reconstituted in dimethyl sulfoxide (DMSO, Sigma). Mevalonate (Sigma) was prepared in sterile-filtered purified water. Human primary neuron cells were obtained from ScienCell Inc. Neuron cell line SH-SY5Y (human) and PC12 (rat) were obtained from American Type Culture Collection. All cells were cultured using the same medium as described in our previous study.\(^14\) All cells were examined for mycoplasma using a MycoAlert Mycoplasma Detection kit (Lonza) prior to experiments.

Conduct of these experiments were not blinded for each operator as measurements were not prone to bias. Deductions from each experimental setup were derived from a collective analysis of all datasets and not just based on a single condition.

\subsection{In vitro ischemic injury model}

In vitro ischemic injury was induced by oxygen–glucose deprivation (OGD) as previously described.\(^15\) Briefly, cells at 80\% confluency were incubated with glucose-free Hank’s balanced salt solution (HBSS) containing 116 mM/L NaCl, 5.4 mmol/L KCl, 0.8 mM/L MgSO\(_4\), 1 mmol/L NaH\(_2\)PO\(_4\), 0.9 mmol/L CaCl\(_2\), and 10 mg/L phenol red (pH 7.4), and were then placed into a modular hypoxia chamber (Billups-Rothenberg) with 95\% N\(_2\) and 5\% CO\(_2\). The chamber was kept in an incubator at 37°C for 6 h to produce OGD. OGD/reperfusion is composed of OGD and reoxygenation period. After OGD, the cultures were removed from the hypoxia chamber and glucose-free HBSS was replaced with media containing Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10\% heat-inactivated fetal bovine serum, 1 mM pyruvate, 1.5 g/L sodium bicarbonate, and placed in a normoxic incubator (reoxygenation) at 37°C.

\subsection{Spheroid culturing}

Cells were suspended as single cells in complete medium containing semi-solid matrix Methylcellulose Base Media (R&D Systems) to a final concentration 1.3\%. Cell mixture at 1 ml (5000 cell/well) were added into six-well plate and incubated for 10 days at 37°C containing 5\% CO\(_2\). After the development of spheroids, drugs suspended in complete medium were added into each well containing spheroids for the following treatment.

\subsection{Treatment}

Cells (primary neuron cells, SH-SY5Y and PC12) were treated with drugs at different concentrations or DMSO. For treatments under normal culturing condition, atorvastatin, lovastatin, cerivastatin, and pitavastatin with or without mevalonate were added and incubated for 48 h. For treatments under OGD condition, atorvastatin, lovastatin, cerivastatin, and pitavastatin with or without mevalonate were added to the cultures 2 h prior to OGD and treatments were continuous. A normal normoxia medium served as the control.

\subsection{Apoptosis assay}

After 48-h drug treatment, the apoptotic cells were determined using Cell Death Detection ELISA kit (Creative diagnostics Inc.) as
per manufacturer’s instructions. This assay is based on the quantitative determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cell lysates using monoclonal antibodies directed against DNA and histones.

2.6 | Measurement of reactive oxygen species (ROS)

After 24-h drug treatment, intracellular ROS level was assessed using live cells and measured using Cellular ROS assay kit (Abcam) as per manufacturer’s protocol. Briefly, cells were stained with DCFDA for 45 min and fluorescence at Ex/Em = 520/605 nm was measured using a fluorometric microplate reader (ThermoFisher Scientific).

2.7 | Measurement of oxidative DNA damage

After 24-h drug treatment, DNA was extracted using DNEasy Mini Kit (Qiagen). 8-Hydroxy-2′-deoxyguanosine (8-OHdG) levels were quantified using the OxiSelect Oxidative DNA Damage ELISA Kit (Cell Biolabs).

2.8 | Measurement of IL-1β and TNF-α levels

After 24-h drug treatment, the release of inflammatory cytokines IL-1β and TNF-α in the supernatant of the cells was determined using ELISA assays according to the kit instructions (R&D Systems).

2.9 | Measurement of Ras activity

After 24-h drug treatment, cellular Ras activity was determined using Ras G-LISA Activation Assay Kit (Cytoskeleton Inc.) according to manufacturer’s instructions.

2.10 | Statistical analyses

Experiments were performed at least three times with triplicate. All datapoints in analyses are biological replicates. PC12 and

![Figure 1](image)

**Figure 1** Influence of different statins on neuron cell survival using cell culturing system. Atorvastatin at 20 and 100 μM (A), pitavastatin at 75 and 100 μM (B), cerivastatin at 25 and 50 μM (C), and lovastatin at 10 and 50 μM (D) significantly induces death of primary human neuron cells, SH-SY5Y and PC12 cells. Cells were treated with statins for 48 h prior to cytoplasmic DNA fragments measurement. *p < .05, compared with control.
SH-SY5Y cells at different passages, and primary human neurons cells from different biological donors, were used in each independent experiment. All datasets were assumed normal and parametric analyses were conducted. The data are expressed as mean and standard deviation. Statistical analyses were performed by unpaired Student’s t-test. A one-way analysis of variance (ANOVA) and the post-hoc Tukey honestly significant difference (HSD) test was also used in the analysis. Values were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | Statins at micromolar concentrations display toxic effects on neuron cells under normal culturing condition

To investigate the biological effects of statin on neurons, we quantified cytoplasmic DNA fragmentation levels in neuron cells after treating with different statins at concentration range spanning from nanomolar to micromolar concentrations. To correlate our findings with clinical relevance, all statins used in our study have been shown to cross the blood brain barrier and into the central nervous system, including atorvastatin, pitavastatin, cerivastatin, and lovastatin.$^{13}$ SH-SY5Y is the most commonly used model to study human neuron cells and PC12 is a rat dopaminergic neural cell line.$^{16}$ SH-SY5Y, PC12, and primary human neuron cells were used to represent in vitro neuron models.

Using the cell culturing system, we found that treatment at 20 and 100$\mu$M atorvastatin significantly increased neuron cell apoptosis compared with control (no drug treatment) ($p < .05$, Figure 1A). Similarly, treatment of neuron cells with pitavastatin (75 and 150$\mu$M), cerivastatin (25 and 50$\mu$M), and lovastatin (10 and 50$\mu$M) resulted in significantly increased cellular apoptosis (Figure 1B–D). In contrast, treatment of neuron cells with atorvastatin (0.1 to 1$\mu$M), pitavastatin (0.5 to 15$\mu$M), cerivastatin (0.05 to 0.5$\mu$M), and lovastatin (0.1 to 1$\mu$M) did not affect cellular viability compared with control. It is noted that statins act on primary human neuron cells, SH-SY5Y and PC12 cells in a similar manner, suggesting that neuron cell lines used in our study represent primary neuron cells. We further applied statins on spheroid culturing system using semi-solid matrix Methylcellulose and found that all tested statins behaved similarly on spheroid culturing system as cell culturing system (Figure 2). Collectively, our results demonstrate that statins at micromolar concentrations have toxic effects on neuron cells under normal culturing condition.

**FIGURE 2** Influence of different statins on neuron cell survival using spheroid culturing system. Atorvastatin at 20 and 100$\mu$M (A), pitavastatin at 75 and 100$\mu$M (B), cerivastatin at 25 and 50$\mu$M (C), and lovastatin at 10 and 50$\mu$M (D) significantly induces death of SH-SY5Y. Spheroids were formed using SH-SY5Y cells on semi-solid matrix methylcellulose. Spheroids were treated with statins for 48h prior to cytoplasmic DNA fragments measurement. *$p < .05$, compared with control.*
### 3.2 Statins at nanomolar concentrations display protective effects on neuron cells under ischemic injury condition

The use of OGD and reperfusion on neuron cells as a model of inducing ischemic injury in vitro is well-established. Consistent with previous work, we also demonstrated that OGD-induced neuron cell death by ~2-fold change compared with control (Figure 3). Interestingly, we found that atorvastatin at the concentrations (0.1 to 1 μM) that did not affect neuron cell viability under normal culturing condition further decreased OGD-induced cell death compared with OGD alone ($p<.05$; Figure 3A). In addition, atorvastatin at the concentrations (20 and 100 μM) that induced neuron cell apoptosis under normal culturing condition further increased OGD-induced cell death compared with OGD alone ($p<.05$; Figure 3A). In contrast, pitavastatin at all tested concentrations did not affect OGD-induced neuron cell death (Figure 3B). Similar to atorvastatin, we showed that cerivastatin at 0.05 to 0.5 μM further decreased and at 25 and 50 μM further increased OGD-induced cell death compared with OGD alone ($p<.05$; Figure 3C). Lovastatin at 0.1 to 1 μM did not affect, and only at 10 and 50 μM further increased OGD-induced cell death compared with OGD alone (Figure 3D).

### 3.3 Statins decreased oxidative stress, oxidative damage, and inflammation-induced by OGD in neuron cells

Ischemia–reperfusion injury involves many complex pathological processes including increasing intracellular ROS, oxidative damage, and releases of inflammatory cytokines; targeting oxidative stress and inflammation prevent ischemia–reperfusion injury. We therefore investigated whether the biphasic effects of statins on OGD-induced neuron cell death were due to their ability in regulating oxidative stress and inflammation. Intracellular ROS and 8-OHdG are known indicators of oxidative stress and oxidative DNA damage, respectively. In line with previous findings, we showed that OGD significantly increased intracellular ROS and 8-OHdG levels compared with control ($p<.05$; Figure 4A,B). In addition, atorvastatin and cerivastatin at concentrations that decreased OGD-induced cell death significantly reversed OGD-induced ROS and 8-OHdG levels. However, atorvastatin and cerivastatin at concentrations that increased OGD-induced cell death did not affect OGD-induced ROS and 8-OHdG levels. Pitavastatin and lovastatin did not affect OGD-induced ROS and 8-OHdG levels compared with OGD alone (Figure 4A,B).

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**Figure 3** The protective effects of different statins on neuron cell survival. (A) Atorvastatin at 0.1, 0.5, and 1 μM significantly decreased OGD-induced neuron cell death whereas at 20 and 100 μM significantly increased OGD-induced cell death. (B) Pitavastatin at all tested concentrations did not affect OGD-induced cell death. (C) Cerivastatin at 0.05, 0.1, and 0.5 μM significantly decreased OGD-induced neuron cell death whereas at 25 and 50 μM significantly increased OGD-induced cell death. (D) Lovastatin at 10 and 50 μM significantly increased OGD-induced cell death. *$p<.05$, compared with OGD.*
OGD significantly increased IL-1β and TNF-α release compared with control ($p < 0.05$; Figure 4C,D). Atorvastatin but not cerivastatin at concentrations that decreased OGD-induced cell death significantly reversed OGD-induced IL-1β level compared with OGD alone ($p < 0.05$; Figure 4C). Interestingly, cerivastatin but not Atorvastatin at concentrations that decreased OGD-induced cell death significantly reversed OGD-induced TNF-α level compared with OGD alone ($p < 0.05$; Figure 4D). Atorvastatin, cerivastatin, and lovastatin at concentrations that further increased OGD-induced cell death did not affect ROS, 8-OHdG, IL-1β, and TNF-α levels in neuron cells (Figure 4).

### 3.4 Statins induces neuron cell apoptosis in a prenylation-dependent manner

Statins have been shown to induce cell death via inhibiting prenylation. Ras activity is known to depend on prenylation. To understand whether the molecular mechanism of pro-apoptotic effect of statins on neuron cells was associated with its capacity to inhibit prenylation, we examined Ras activity after statins treatment. We found that atorvastatin, pitavastatin, and lovastatin significantly decreased Ras activity in neuron cells ($p < 0.05$; Figure 5A). In contrast, cerivastatin did not affect Ras activity. Consistently, the addition of mevalonate, a substrate of HMG-CoA reductase, completely abrogated the pro-apoptotic effect of atorvastatin, pitavastatin, and lovastatin but not cerivastatin in neuron cells ($p < 0.05$; Figure 5B). However, the addition of mevalonate did not affect the effects of atorvastatin, pitavastatin, lovastatin, and cerivastatin on neuron cell ROS level under ischemic injury condition (Figure 5C). Similarly, mevalonate did not affect the ability of atorvastatin and cerivastatin in reducing OGD-induced release of IL-1β and TNF-α, respectively ($p < 0.05$, Figure 5D,E). These results demonstrate that (1) atorvastatin, pitavastatin, and lovastatin induce neuron cell apoptosis via prenylation-dependent manner; (2) other mechanisms are involved in the pro-apoptotic effect of cerivastatin; (3) prenylation is not involved in the protective effects of statins under ischemic injury condition.

### 4 DISCUSSION

Although statins are indicated in the prevention of cardiovascular risk, substantial evidence from recent clinical studies highlight

![Figure 4](image-url)  
**Figure 4** Influence of different statins on OGD-induced neuron cell oxidative stress, oxidative DNA damage, and inflammatory response. Atorvastatin and cerivastatin but not pitavastatin or lovastatin significantly reversed OGD-increased ROS (A) and 8-OHdG (B) level in neuron cells. (C) Atorvastatin but not other statins significantly reversed OGD-induced IL-1β level in neuron cells. (D) Cerivastatin but not other statins significantly reversed OGD-induced TNF-α level in neuron cells. A, B, C, D, and E represent 0.1, 0.5, 1, 20, and 100 μM for atorvastatin; 0.5, 2.5, 15, 75, and 150 μM for pitavastatin; 0.05, 0.1, 0.5, 25, and 50 μM for cerivastatin; 0.1, 0.5, 1, 10, and 50 μM for lovastatin. *$p < 0.05$, compared with OGD.
statins penetrate the blood brain barrier and might have pharmacological effects within the central nervous system.\textsuperscript{26} Majority of studies are focusing on individual statins using in vitro and limited in vivo neurological or neurological-like models to analyze potential effects and mechanisms through which particular statin may exert its neurological action. Of note, the results of clinical and preclinical studies are conflicting and inconclusive to date: both beneficial effect and statin-associated neurological detriment are reported.\textsuperscript{6–8,10–12} The reason behind this discrepancy is unclear, and may be a result of differences in model systems and statin concentrations. In this work, we compared how four clinically relevant, and structurally and pharmacologically diverse statin compounds affect neuronal viability under normal culturing and OGD-induced ischemic injury conditions.

To demonstrate the biological effects of statins and clinical relevance, we used three different types of neuron cells, including human primary neuron cells, neuron cell lines from human and rat species. Although SH-SY5Y was derived from a patient with neuroblastoma, it is commonly used as the representative neuron model because of its human origin, ease of maintenance, and catecholaminergic neuronal properties.\textsuperscript{16} We observed a biphasic effect of statins on neuron cell viability under normal culturing and OGD-induced ischemic injury conditions: being protective to reverse OGD-induced cell death at low, nanomolar concentrations, and this protective effect disappeared at higher concentrations. Under normal culturing condition, statins at low nanomolar concentrations did not affect cell viability whereas at high micromolar concentrations, this significantly induced cell apoptosis (Figures 1 and 2). To our knowledge, only two studies from Dukie Anoopkumar’s laboratory have compared the effects of multiple statin compounds (atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin) within a single in vitro model of neuroinflammation on a microglial-like cell line. They demonstrated that atorvastatin, pravastatin, and rosuvastatin protected neuron cells from lipopolysaccharide-induced decrease in cellular viability.\textsuperscript{27,28} In agreement with their findings, we showed that atorvastatin also protected neuron cells from OGD-induced cell death. We noted that all four statins induced apoptosis at higher

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 5** Influence of different statins on neuron cell prenylation. (A) Atorvastatin, pitavastatin, and lovastatin but not cerivastatin significantly decreased Ras activity in neuron cells. A and B represent 20 and 100μM for atorvastatin; 75 and 100μM for pitavastatin; 25 and 50μM for cerivastatin; 10 and 50μM for lovastatin. (B) The addition of mevalonate (MV) significantly reversed the pro-apoptotic effects of atorvastatin, pitavastatin, and lovastatin but not cerivastatin. Atorvastatin at 100μM, pitavastatin at 100μM, cerivastatin at 50μM, and lovastatin at 50μM were used. There was no difference on OGD-induced ROS level (C), IL-1β level (D), and TNF-α level (F) between mevalonate group and mevalonate + statin group. Atorvastatin at 1μM, pitavastatin at 15μM, cerivastatin at 0.5μM, and lovastatin at 1μM were used. *\(p < .05\), compared with control.
concentrations whereas only atorvastatin and cerivastatin demonstrated protective effects at lower concentrations. This suggests the differential effects on neuron cells among statins. This agrees with clinical studies demonstrating that statin-associated clinical effects and pharmacological characteristics are different among statins. We speculate that it might be because statins are structurally diverse. The clinically recommended dosage of atorvastatin, pitavastatin, cerivastatin, and lovastatin are 10–80 mg, 2–4 mg, 0.1–0.3 mg, and 10–80 mg per day for patients with dyslipidemia. The mean serum concentration of atorvastatin and cerivastatin is <50 nM in patients after a single clinically recommended dose. According to our in vitro findings, the effective concentrations of statins were from 50 nM (Figures 1–3). Although these concentrations of statins are higher than achievable in current therapeutic use, our work provides a proof-of-concept that addition of atorvastatin and cerivastatin can alleviate OGD-induced toxicity in neuron cells. Our findings suggest that alternative administration routes of atorvastatin and cerivastatin will be required to increase its serum concentration to prevent OGD-induced neuron injury.

As expected, their underlying mechanisms vary among individual statins. Generally, our mechanism studies demonstrated that atorvastatin, pitavastatin, and lovastatin induced neuron cell death via inhibiting prenylation (Figure 5A,B). In contrast, statins reversed OGD-induced cell death via alleviating oxidative stress and damage, and inflammatory response; and furthermore this is independent of prenylation (Figures 4 and 5C–E). Our findings are consistent with many studies that show protective effects are often associated with reduced neuroinflammatory mediator release and oxidative stress. Although statins decreased OGD-induced inflammation, atorvastatin acted on it via decreasing IL-1β without affecting TNFα whereas cerivastatin acted on it via decreasing TNFα without affecting IL-1β. In addition, cerivastatin did not induce neuron cell death via inhibiting prenylation (Figure 5A,B). These further confirm that individual statins exhibit differing pharmacological characteristics in neuron cells.

In conclusion, our work shows the biphasic influence of statins on neuron models under normal culturing and pathological conditions (Figure 6). Findings from the present study highlight the need to consider individual statins as having discrete effects within the central neuron system, with potentially varied mechanism of action. Our work supports clinical trials to establish the relevance of these pleiotropic effects of statins to human neurological and neurodegenerative disorders.

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DATA AVAILABILITY STATEMENT
Datasets that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
Not applicable.

DISCLOSURE
All authors declare that they no conflict of interest.

ORCID
Zhenli Guo https://orcid.org/0000-0002-8657-7825
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