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Atorvastatin Rejuvenates Neural Stem Cells Injured by Oxygen–Glucose Deprivation and Induces Neuronal Differentiation Through Activating the PI3K/Akt and ERK Pathways

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Abstract

Oxygen and glucose (OGD) deprivation is one of the most important pathogenic mechanisms in cerebral infarction and is widely used as an in vitro model for ischemic stroke. OGD also damages neural stem cells (NSCs), which are important in brain recovery after cerebral infarction. To enhance recovery, there have been many studies aimed at determining methods to protect NSCs after stroke. Because atorvastatin has diverse protective effects on neural cells, we studied whether it could rejuvenate NSCs injured by OGD. Primary cultured NSCs were exposed to OGD for 8 h, and the main characteristics of stem cells, such as survival, proliferation, migration, and differentiation, were evaluated to confirm the effect of OGD on NSCs. Next, cells were treated with various concentrations of atorvastatin with exposure to OGD for 8 h to confirm whether it could rejuvenate NSCs. OGD significantly affected the survival, proliferation, migration, and differentiation of NSCs. However, treatment with atorvastatin meaningfully restored survival, proliferation, migration, and differentiation of NSCs. These beneficial effects of atorvastatin were blocked by treatment with either a PI3K inhibitor or an ERK inhibitor. In conclusion, OGD damages NSCs and causes them to lose the main characteristics of stem cells so that they cannot contribute to brain recovery after cerebral infarction. However, treatment with atorvastatin after cerebral infarction can effectively rejuvenate NSCs through activating the PI3K and ERK pathways to aid in brain regeneration.

Keywords Stroke · Oxygen–glucose deprivation · Atorvastatin · Neural stem cells · Phosphatidylinositol 3-kinase · Extracellular signal-regulated kinase

Introduction

With the growing aging population, the number of patients with cerebral infarction is rapidly increasing. Therefore, finding ways to prevent cerebral infarction and restore its neurologic sequelae is of worldwide interest. Although numerous studies have been performed for this purpose, there is no definite way to prevent and/or cure cerebral infarction. Based on the results obtained from basic research on cerebral infarction, many pathogenic mechanisms are involved. Deprivation of oxygen and glucose (OGD) is one of the most well-known pathogenic mechanisms of cerebral infarction [1]. Cerebral infarction can occur when cerebral blood flow abruptly stops, which leads to OGD in the brain and eventually damages both neurons and many other supporting cells. For this reason, great effort has been made to protect the brain against OGD. Additionally, OGD can be used as an in vitro model of cerebral infarction [2].

Na-Young Choi and Ji Young Kim contributed equally to this work.

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Neural stem cells (NSCs) are known to exist in the adult brain and are involved in regeneration of the brain, although this process is very limited. Considering that the brain is damaged in cerebral infarction, regeneration of the damaged brain by NSCs could be extremely important in reducing the neurologic sequelae after cerebral infarction. A previous report indirectly supported this hypothesis by showing that patients with an intact subventricular region of the lateral ventricle, which is where NSCs are located, had better outcomes than patients with a damaged subventricular region after cerebral infarction [3]. This indicates that NSCs preserved from damage during cerebral infarction can contribute to regeneration of the damaged brain and recovery from neurologic sequelae. However, cerebral infarction and OGD also damage NSCs, so it is important to find ways to protect NSCs against OGD.

Atorvastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, is used for the treatment of hyperlipidemia and for the prevention of events associated with cardiovascular disease. It is also known to have neuroprotective effects against various stressful conditions through diverse mechanisms. Therefore, atorvastatin is widely used in patients with cerebral infarction. In our previous study, we confirmed that atorvastatin protects motor neurons from oxidative stress [4]. Other studies from two different groups showed that treatment with atorvastatin improved recovery after cerebral infarction [5, 6]. In particular, Chen et al. reported that atorvastatin increases neural progenitor cell proliferation after stroke by promoting presenilin-1 expression and Notch1 activity [5]. Taken together, these previous findings indicate that atorvastatin can protect the brain after cerebral infarction. However, the effects of atorvastatin on the characteristics of NSCs damaged by cerebral infarction and OGD have not yet been established.

In the present study, we examined how OGD affects the characteristics of NSCs, whether atorvastatin can rejuvenate NSCs damaged by OGD, and the mechanisms involved in the rejuvenation of NSCs by atorvastatin.

Materials and Methods

Primary Culture of NSCs

All animal procedures were conducted in accordance with Hanyang University's guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Hanyang University. All efforts were made to minimize the number of animals used and animal suffering. All animals in the study were only used once.

Neural stem cells (NSCs) were isolated from embryonic rodent brains, cultured, and expanded. The protocol for NSC culture was described previously and has been widely used in previous studies [7–10]. Rat embryos (Sprague–Dawley rats; Orient Bio, Seongnam, South Korea) were decapitated at embryonic day 13 (E13), and the brains were swiftly removed and placed in a Petri dish with ice-cold Hank's balanced salt solution (HBSS 3.9 g/l HEPES; Gibco, Invitrogen Corporation, Grand lsland, NY, USA). Embryonic brain tissue was dissected from the cortex. Single cells were plated at a density of 20,000 cells/cm^2 on culture dishes precoated with poly-L-ornithine/fibronectin in phosphate-buffered saline (PBS) and were cultured in N2 medium (DMEM/F12, 25 mg/l insulin, 100 mg/l transferrin, 30 nM selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, 0.2 mM ascorbic acid, 8.6 mM D(+)glucose, 20 nM $NaHCO_3$, and 1% penicillin/streptomycin) supplemented with basic fibroblast growth factor (BFGF; 10 ng/ml, R&D Systems, Minneapolis, MN, USA) for 5–6 days as a monolayer on the adherent surface. The cultures were maintained at 37 °C in a humidified 5% CO_2 incubator.

To confirm the identity of NSCs, cells were seeded on chamber well plates at a density of 1×10^5 cells/well for 24 h. The cells were fixed in 2% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 20 min. The cells were incubated with 5% normal serum in PBS for 60 min and then incubated overnight in PBS with 2% normal serum and mouse anti-nestin primary antibodies (1:100; Abcam, Cambridge, UK). The next day, the cells were incubated for 60 min in 2% normal serum in PBS containing tetramethylrhodamine goat anti-rabbit lgG (H + L) secondary antibodies (Life Technologies, Carlsbad, CA, USA). The cells were washed several times with PBS, mounted on glass slides with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA), and observed with a fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan) using the appropriate excitation and emission wavelengths.

To determine if the primary cultured NSCs could be differentiated into diverse neural cells, differentiation of NSCs into neurons and astrocytes was induced and confirmed by the following procedures. First, NSCs were seeded in coating plates with culture media. On the next day, the N2 media was removed and replaced with rat NSC differentiation media (Cell Applications, Inc., San Diego, CA, USA), and the differentiation media was changed every 2–3 days. After 9 and 11 days, the differentiation of NSCs into neurons and astrocytes was confirmed by immunocytochemistry using the following primary antibodies: nestin (1:100, Millipore, Bedford, MA, USA), doublecortin (1:100, Cell signaling Technology, Beverly, MA, USA), and GFAP (1:100, Abcam). On the next day, the cells were washed and incubated with secondary antibodies for 1 h. Tetramethylrhodamine goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA), Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes),

tetramethylrhodamine goat anti-mouse IgG antibody (Molecular Probes), and Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes) were used as secondary antibodies. Finally, the cells were mounted with DAPI mounting solution (Vector Laboratories Inc.). Throughout the procedure, the cells were washed out three times with DPBS between each step. Immunocytochemistry was performed as described above (Fig. 1a).

Exposure of NSCs to OGD and Atorvastatin Treatment

To determine the effect of oxygen–glucose deprivation (OGD) on the characteristics of NSCs, the cells were exposed to OGD. All OGD experiments were conducted in an anaerobic chamber. A gas mixture containing CO_2 (5%), O_2 (0.2%), and N_2 (94.8%) was flushed through the chamber for 0–24 h. This process preserved an environment of non-fluctuating hypoxia below 1% O_2 [11].

Atorvastatin was a generous gift from Chong Kun Dang Pharm, Inc. Atorvastatin was dissolved in dimethyl sulfoxide (DMSO) before use, and the final concentration of DMSO in culture media was 1% (vol/vol). To evaluate the effect of atorvastatin on NSCs, the cells were treated with different concentrations of atorvastatin (0, 0.01, 0.1, 1, 10, and 100 μM) in glucose-free N2 medium for 24 h. To determine if atorvastatin could rejuvenate NSCs damaged by OGD, NSCs were treated with atorvastatin with exposure to OGD for 8 h.

MTT, Trypan Blue Staining, and Lactate Dehydrogenase Assay

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide (MTT; Sigma, Saint Louis, MO, USA). NSCs were seeded on 96-well plates and treated with atorvastatin (0, 0.01, 0.1, 1, and 10 μM) with exposure to OGD for 8 h. After incubation, MTT solution was added to each well and incubated for 2 h. Finally, the MTT solution was removed from each well, and 200 μl of dimethyl sulfoxide was added. Cell viability was measured using an ELISA reader (Synergy H1 Hybrid reader, BioTek, Winooski, VT, USA) at 540 nm.

For trypan blue staining, 10 μl of cells was incubated for 2 min with 10 μl of trypan blue solution (Gibco). Unstained live cells were counted with a hemocytometer.

A colorimetric assay kit (Roche Boehringer-Mannheim, Indianapolis, IN, USA) was used to quantify lactate dehydrogenase (LDH) released from cultured NSCs according to the manufacturer's instructions. Cytotoxicity was measured using an ELISA reader (Synergy H1 Hybrid reader) at 490–690 nm.

TUNEL Staining for Evaluation of Apoptosis

Apoptosis of cells exposed to OGD and treated with different concentrations of atorvastatin for 8 h was evaluated using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay according to the manufacturer's protocol (Roche Boehringer-Mannheim). The cells were fixed with 4% paraformaldehyde in DPBS for 1 h and incubated with 3% H_2O_2 in methanol for 10 min. Cells were then washed with DPBS and incubated with 0.5% Triton X-100 in 0.1% sodium citrate for 2 min. To monitor intact, condensed, and fragmented nuclei, the TUNEL-stained cells were washed several times with PBS for 2 min and mounted on glass slides with mounting medium containing DAPI (Vector Laboratories). The cells were observed under a fluorescence microscope (Eclipse Ti, Nikon) with the appropriate excitation and emission wavelengths for DAPI and TUNEL staining.

Immunostaining for nestin and Ki-67

To assess the effects of OGD and atorvastatin on the characteristics of NSCs, primary cultured NSCs were seeded at 1×10^5 cells on a 4-well slide glass plate and treated with atorvastatin (0, 0.01, 0.1, 1, and 10 μM) with exposure to OGD for 8 h. The cells were fixed with 2% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 15 min, permeabilized with 0.5% Triton X-100 in DPBS for 5 min, and washed several times with DPBS for 5 min. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in DPBS for 20 min. The cells were incubated with 5% normal serum in DPBS for 60 min and then incubated overnight with 2% normal serum in DPBS at 4 °C with the following primary antibodies: mouse anti-nestin (1:100; Abcam) and rabbit anti-Ki-67 (1:100; Abcam). The next day, the cells were incubated in DPBS containing 2% normal serum and tetramethylrhodamine goat anti-rabbit lgG (H + L) (Life Technologies) and goat anti-mouse Alexa Fluor 488 (Life Technologies) secondary antibodies for 60 min, washed three times with DPBS, and mounted on glass slides using DAPI mounting solution (Vector Laboratories). The cells were imaged under a fluorescence microscope (Eclipse Ti, Nikon) at the appropriate excitation and emission wavelengths.

BrdU Cell Proliferation Assay

To measure the proliferation of NSCs, cells exposed to OGD and treated with different concentrations of atorvastatin (0, 0.01, 0.1, 1, and 10 μM) for 8 h, NSCs were labeled with 10 μM BrdU for 16 h, and cell proliferation was determined using a BrdU Labeling and Detection Kit (Roche Boehringer-Mannheim) according to the manufacturer's instructions. Cell

proliferation was measured using an ELISA reader (Synergy H1 Hybrid reader) at 370–492 nm.

Colony Forming Unit Assay

The proliferation of NSCs was calculated via the colony forming unit (CFU) assay. Approximately 8×10^3 cells were seeded in a 60-mm grid plate and treated with atorvastatin (0, 0.01, 0.1, 1, and 10 μM) with exposure to OGD for 8 h. The cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and the culture media was replaced. After 14 days, the cells were washed again with DPBS and stained with 0.5% crystal violet (Sigma) in methanol for 30 min at room temperature. After staining, the plates were washed with DPBS and allowed to dry. Colony counting was performed using a dissecting microscope, and colonies that were less than 2 mm in diameter or faintly stained were excluded.

Migration Assays

Cell migration assays were performed using a QCM 24-well colorimetric cell migration assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, NSCs (3×10^5 cells) were seeded in the upper chamber and different concentrations of atorvastatin (0, 0.01, 0.1, 1, and 10 μM) were treated into the lower chamber. The cells were exposed to OGD for 8 h at 37 °C. The NSCs that had migrated through the membrane were stained, and the number of migrated cells was determined using a spectrophotometer plate reader (Synergy H1 Hybrid reader) at an absorbance of 560 nm [12].

FACS Analysis for Evaluating NSC Differentiation

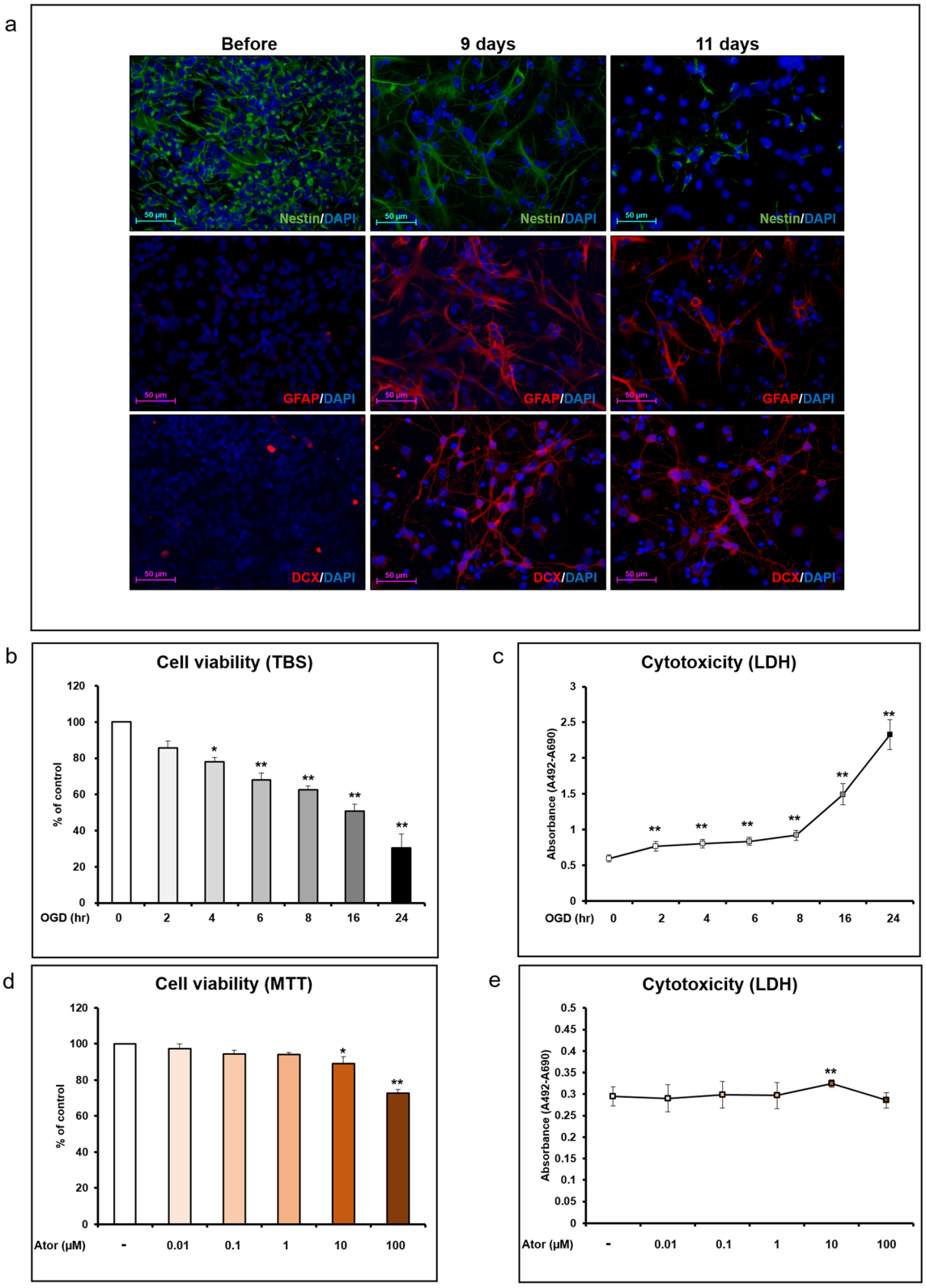
To determine if the differentiation of NSCs into neurons and astrocytes was affected by exposure to OGD for 8 h and atorvastatin treatment at different concentrations, flow cytometry analysis was performed. NSCs exposed to diverse conditions were washed twice and fixed with 2% paraformaldehyde. The tubes were centrifuged at 300x*g* for 5 min, and the pellets were permeabilized with 0.1% Triton X-100. The pellets were then incubated with specific primary antibodies. The antibodies used in these experiments were as follows: doublecortin (1:100, Cell Signaling Technology), GFAP-FITC (1:20, eBioscience Inc.), and nestin-PE (1:20, BD Pharmingen). After primary antibody incubation, the cells were centrifuged at 300x*g* for 5 min and incubated for 1 h with goat anti-rabbit Alexa Fluor 488 (Life Technologies) as a secondary antibody. Finally, the cell solutions were analyzed using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and positive cells were analyzed using BD Accuri C6 software.

Western Blotting

Levels of PI3K, phosphorylated Akt (pAkt; Ser-473), phosphorylated glycogen synthase kinase-3β (GSK-3β; Ser-9), B cell lymphoma-2 (Bcl-2), Ki67, Bax, cytosolic cytochrome C, cleaved caspase-9, cleaved caspase-3, extracellular signal-regulated kinase (ERK), pERK (Thr202/204), p38, and p-p38 (Thr180/Tyr182) were analyzed by Western blotting. Briefly, 5×10^6 cells were washed twice in cold PBS and incubated in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% sodium dodecyl sulfate (SDS), 100 mg/ml phenylmethylsulfonyl fluoride (PMSF), 50 ml/ml aprotinin, 1% Igepal 630, 100 mM NaF, 0.5% sodium deoxycholate, 0.5 mM EDTA, 0.1 mM EGTA] for 30 min on ice. The unbroken cells and nuclei were pelleted by centrifugation for 15 min at 16,200×*g*, and the lysates were cleared by centrifugation at 10,000×*g*.

Mitochondrial and cytosolic fractions were isolated using a Mitochondria/Cytosol Fractionation Kit (Abcam) according to the manufacturer's instructions for evaluation of cytosolic cytochrome C level. Briefly, after treatment with atorvastatin (0, 0.01, 0.1, and 1 μM) and exposure to OGD for 8 h, the NSCs were harvested, washed once with ice-cold PBS, and resuspended in 1.0 ml of 1× cytosol extraction buffer mix containing dithiothreitol (DTT) and protease inhibitors. After incubation on ice for 10 min, the cell suspension was homogenized by passing through a syringe 30 to 50 times. The samples were centrifuged at 900×*g* at 4 °C for 10 min. The supernatants were centrifuged again at 16,200×*g* for 30 min to separate the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant).

Samples containing equal amounts (40 mg) of protein were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked using 2% milk before incubation with specific primary antibodies against PI3K (1:500), Akt (1:1000), pAkt (Ser-473) (1:100), GSK-3β (1:1000), pGSK-3β (Ser-9) (1:200), Bcl-2 (1:200), Bax (1:500), cytosolic cytochrome C (1:200), cleaved caspase-9 (1:200), cleaved caspase-3 (1:200), ERK (1:1000), pERK (1;500), p38 (1:1000), p-p38 (Thr180/Tyr182) (1:600) (all from Cell Signaling Technology), and Ki67 (1:200; Abcam). The membranes were washed with Tris-buffered saline containing 0.1% Tween-20, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Jackson Immunoresearch Laboratories, Bar Harbor, ME, USA). The membranes were then stained and visualized using a West-Q Chemiluminescent Substrate Plus Kit (GenDEPOT, Katy, TX, USA). The Western blots were quantified using an image analyzer (ImageQuant LAS 4000; GE Healthcare, Little Chalfont, UK).


a
Before
9 days
11 days
Nestin/DAPI
GFAP/DAPI
DCX/DAPI
50 μm
b
Cell viability (TBS)
% of control
OGD (hr)
0
2
4
6
8
16
24
c
Cytotoxicity (LDH)
Absorbance (A492-A690)
d
Cell viability (MTT)
Ator (μM)
-
0.01
0.1
1
10
100
e
Cytotoxicity (LDH)

◀ **Fig. 1** Effects of oxygen–glucose deprivation (OGD) and atorvastatin on neuronal stem cell (NSC) viability and cytotoxicity. Immunocytochemistry shows that the cells cultured in our system are positive for nestin, a well-known neural stem cell marker, and can differentiate into DCX-positive cells indicating neurons or GFAP-positive cells suggesting astrocytes when incubated under the appropriate conditions. These findings show that the primary cultured cells in our system are neural stem cells (**a**). NSCs were incubated in an anaerobic chamber with glucose-free N2 medium for different exposure times (0–24 h). Cell viability significantly decreased and cytotoxicity significantly increased in a time-dependent manner after exposure to OGD (**b**, **c**). NSCs were treated with several concentrations of atorvastatin without OGD. Atorvastatin treatment alone did not affect the viability of NSCs up to 1 μM (**d**, **e**). Data are presented as the mean (% of control) ± SD. Treatment groups were compared with the control group using one-way ANOVA followed by Tukey's test (**a** $n = 4$, **b** $n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group)

Inhibition of the PI3K and ERK Pathways

To investigate whether the PI3K and ERK pathways play critical roles in the effects of atorvastatin on the rejuvenation of NSCs, we pretreated cells with a PI3K inhibitor, LY294002 (Sigma), or an ERK inhibitor, FR180204 (Millipore), before exposure to various conditions. Cell viability and cytotoxicity were assessed by the MTT assay and LDH assay, respectively. NSCs were separated into ten groups as follows: control (group 1), 8-h OGD alone (group 2), 8-h OGD + 0.1 μM atorvastatin (group 3), 8-h OGD + 1 μM atorvastatin (group 4), 10 μM LY294002 or FR180204 + 8-h OGD + 0.1 μM atorvastatin (group 5), 10 μM LY294002 or FR180204 + 8-h OGD + 1 μM atorvastatin (group 6), 8-h OGD + 10 μM LY294002 or FR180204 (group 7), 10 μM LY294002 or FR180204 + 0.1 μM atorvastatin (group 8), 10 μM LY294002 or FR180204 + 1 μM atorvastatin (group 9), and 10 μM LY294002 or FR180204 alone (group 10).

Statistical Analyses

All data are expressed as the mean ± SD of five or more independent experiments. Statistical comparisons of viability, cytotoxicity, proliferation, and Western blot data between different treatment groups were performed using two-way ANOVA followed by Tukey's test. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS 17.0 software package for Windows (SPSS, Seoul, Korea).

Results

OGD Significantly Increases NSC Death, Which Is Effectively Reduced by Atorvastatin Treatment

After confirming that the primary cultured cells in our system were neural stem cells (NSCs) (Fig. 1a), the effect of oxygen–glucose deprivation (OGD) on NSC damage was determined by exposing primary cultured NSCs to OGD for 2, 4, 6, 8, 16, and 24 h, which significantly decreased NSC viability in a time-dependent manner (Fig. 1b, c). The optimal duration of OGD for subsequent experiments was determined to be 8 h because cell viability was 60–70% under this condition (Fig. 1b, c).

Next, to evaluate the effect of atorvastatin on NSCs, cells were treated with a range of concentrations of atorvastatin for 24 h, and it was confirmed that atorvastatin alone had no significant effect on NSCs up to concentrations of 1 μM (Fig. 1d, e). Based on these findings and the fact that an atorvastatin concentration greater than 10 μM cannot be achieved in human serum, concentrations of 0.01, 0.1, 1, and 10 μM atorvastatin were used for further studies.

When examining whether atorvastatin could rescue NSCs from OGD, atorvastatin meaningfully reduced NSC death induced by OGD (Fig. 2a–c). In detail, 8-h OGD decreased the viability of NSCs by about 30%, while atorvastatin treatment from 0.1 to 1 μM significantly increased NSC viability based on results from the MTT, TBS, and LDH assays.

Additionally, to assess changes in apoptosis, TUNEL staining was performed and showed that the number of TUNEL-positive cells was highly increased by 8-h OGD but was decreased by atorvastatin treatment up to 1 μM in a concentration-dependent manner (Fig. 3a, b).

The Proliferative Capacity of NSCs Is Inhibited by OGD but Is Recovered by Atorvastatin Treatment

One of the most important characteristics of stem cells is their proliferative capacity, so we evaluated the effect of OGD on the proliferation of NSCs. As shown in Fig. 4a, OGD significantly reduced the expression of Ki67 in NSCs, which is a proliferation marker. However, treatment with atorvastatin up to 1 μM dose-dependently restored Ki67 level (Fig. 4a). Using a BrdU assay to measure the proliferative activity of NSCs, OGD markedly decreased the number of BrdU-positive cells, while atorvastatin treatment up to 1 μM significantly increased the BrdU-positive cell number (Fig. 4b). The colony forming unit assay was also performed to directly evaluate the proliferation of NSCs. OGD decreased the number of colony cells and colony formation, while atorvastatin refreshed NSCs and significantly increased colony cell number and formation (Fig. 4c–e).

The Migratory Capacity of NSCs Is Diminished by OGD but Is Recovered by Atorvastatin Treatment

Another important characteristic of stem cells is their migratory capacity. To confirm whether OGD affects the migration of NSCs, cell migration assay kits were used and showed that OGD highly damaged the migratory capacity of NSCs compared with the control (Fig. 5).

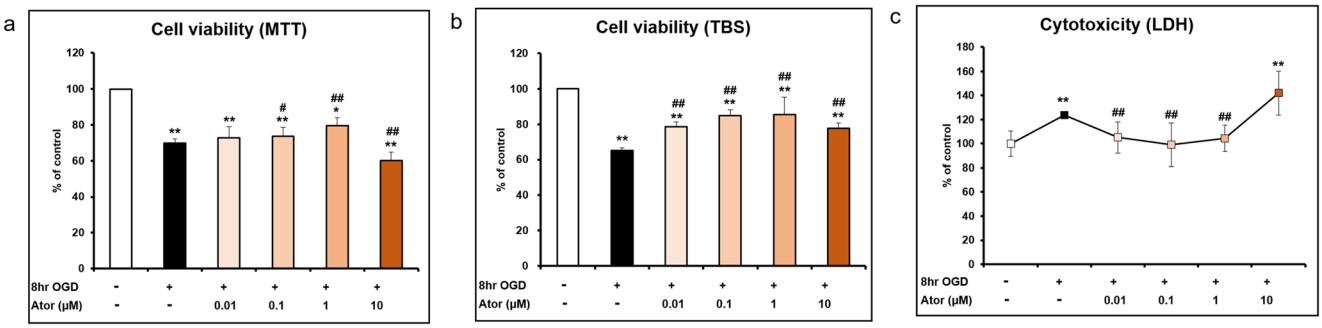


Fig. 2 Effect of atorvastatin on the viability and cytotoxicity of neuronal stem cells (NSCs) after treatment with 8-h OGD and/or atorvastatin. Exposure to 8-h OGD reduced the viability of NSCs by about 35% (**a**, **b**) and increased cytotoxicity to around 25% (**c**). However, atorvastatin effectively restored the viability of NSCs in a concentration-dependent manner up to 1 μM. Data are presented as the mean (% of control) ± SD. Treatment groups were compared with the control group using two-way ANOVA followed by Tukey's test (**a** $n = 4$, **b** $n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone)

However, the migratory capacity of NSCs was significantly revived with atorvastatin treatment (Fig. 5).

OGD Significantly Induces Glial Differentiation of NSCs, While Atorvastatin Increases Neurogenesis and Reduces Gliogenesis in NSCs Exposed to OGD

Differentiation capacity is also an important characteristic of NSCs. NSCs can naturally differentiate into diverse neural cells such as neurons and astrocytes. To determine if OGD and atorvastatin affect the differentiation of NSCs, FACS analyses were performed with several different antibodies for identification of neurons and astrocytes. FACS staining with neuron- and astrocyte-specific antibodies indicated that OGD significantly increased glial differentiation of NSCs, while atorvastatin markedly induced neuronal differentiation but reduced glial differentiation of NSCs exposed to OGD (Fig. 6).

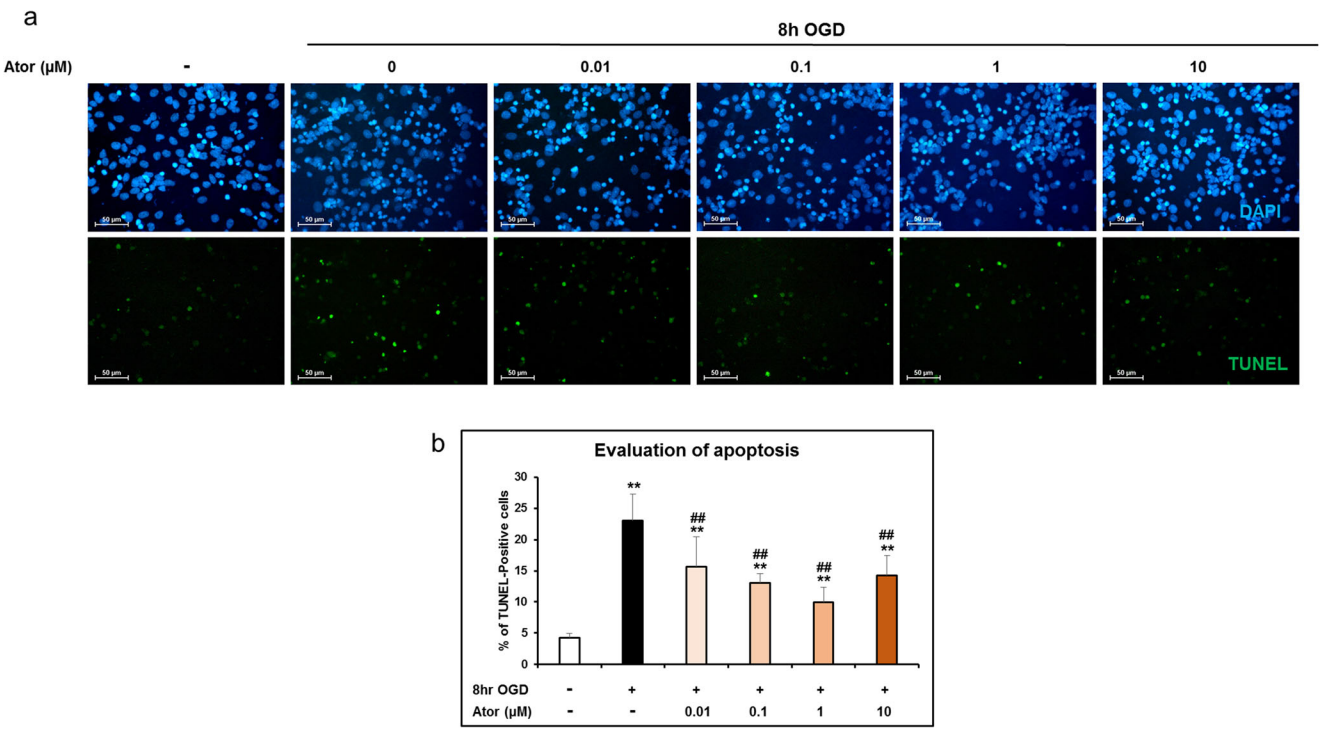


Fig. 3 Anti-apoptotic effect of atorvastatin on NSCs treated with 8-h OGD. NSCs were labeled by TUNEL staining (**a**). Apoptosis of NSCs markedly increased with exposure to 8-h OGD but significantly decreased with atorvastatin treatment in a concentration-dependent manner (**b**). Data are presented as the percentage of TUNEL-positive cells ± SD. Treatment groups were compared with the control group using two-way ANOVA followed by Tukey's test ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone)

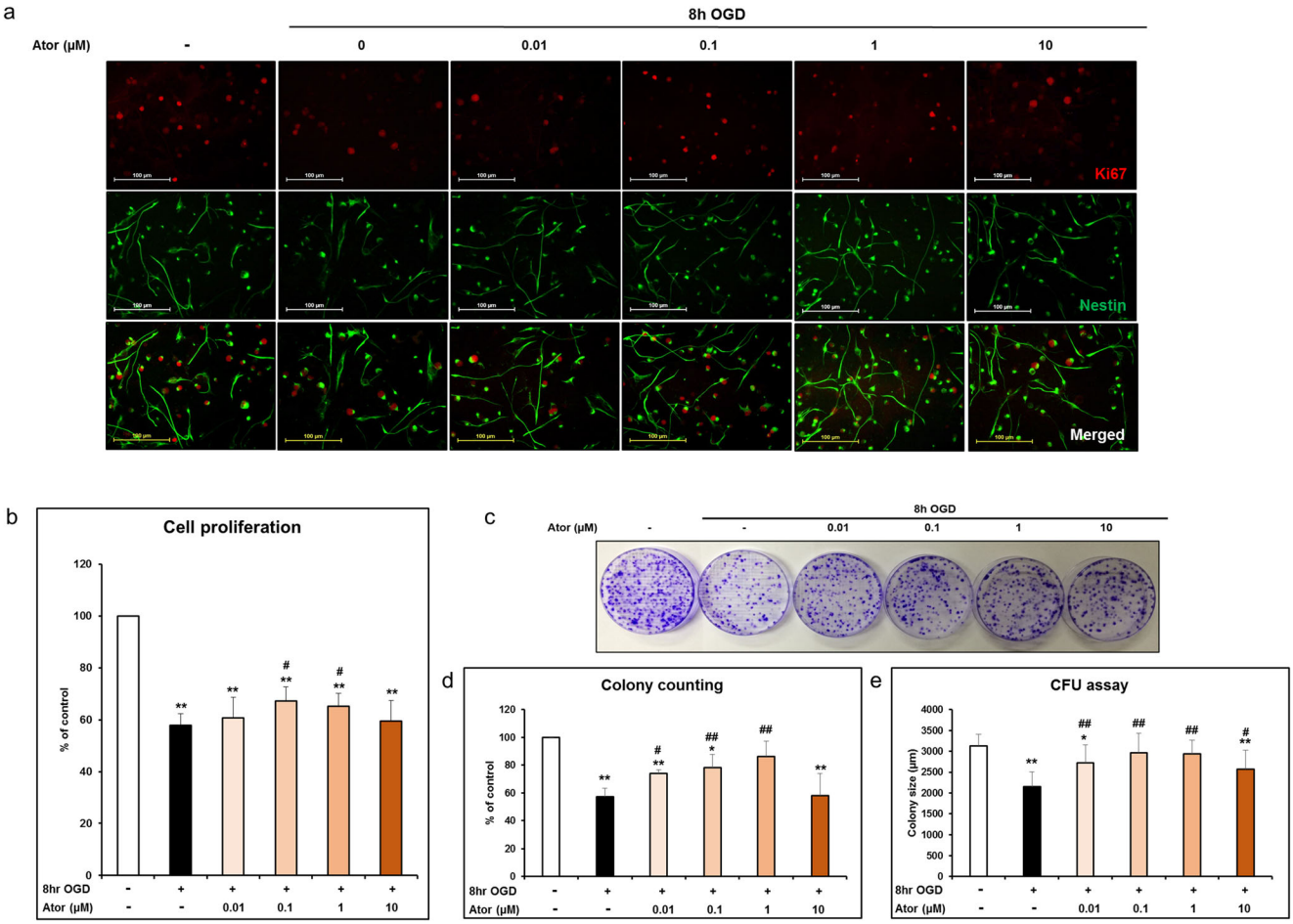


Fig. 4 Effect of atorvastatin on the proliferation of NSCs after 8-h OGD. Immunostaining of Ki-67 and nestin, markers of proliferative activity in NSCs (**a**), BrdU assay (**b**), and colony forming unit assay (**c**–**e**) demonstrated that treatment with atorvastatin significantly restored the proliferation of NSCs that was inhibited by 8-h OGD. Data are presented as the mean (% of control) ± SD of five independent experiments. Treatment groups were compared with the control group using two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone)

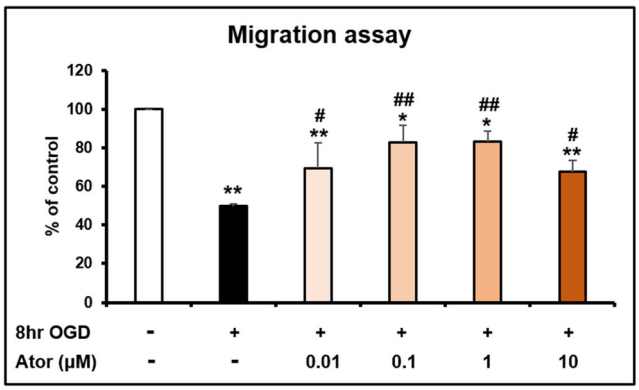
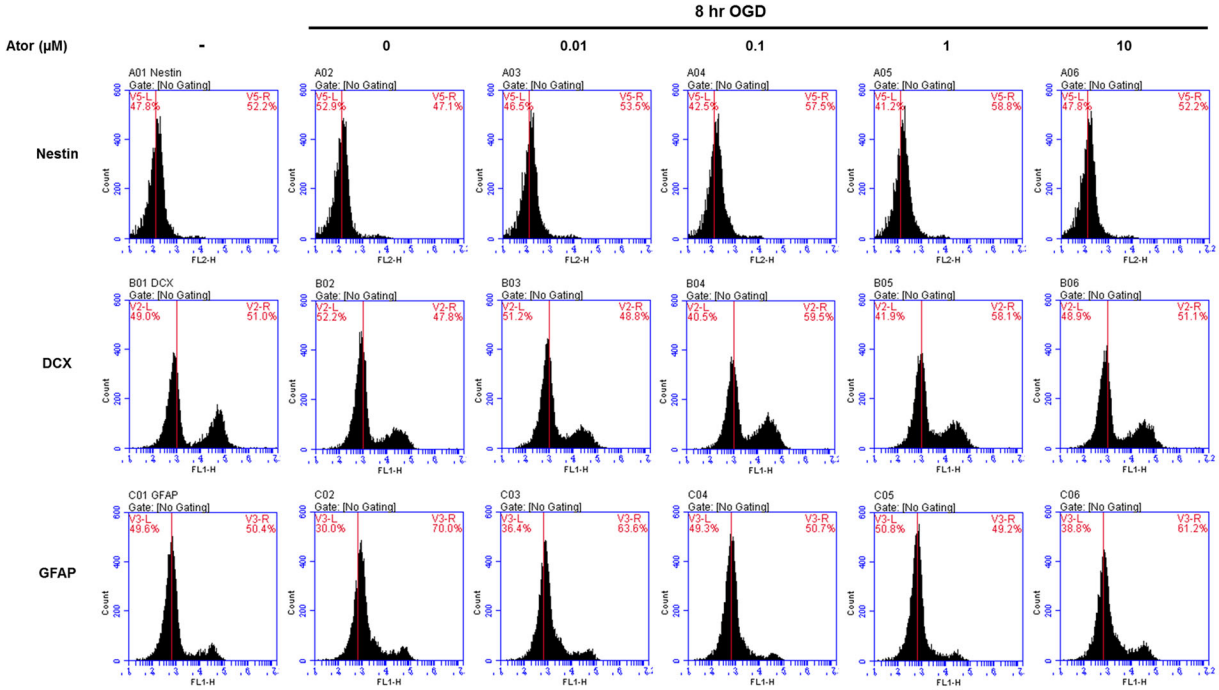


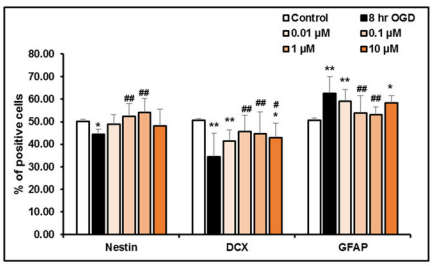
Fig. 5 Effect of OGD and atorvastatin on the migratory capacity of NSCs. Migration was decreased by about 50% after exposure to 8-h OGD, but atorvastatin treatment efficiently recovered the migratory capacity of NSCs. Data are presented as the mean (% of control) ± SD of five independent experiments. Treatment groups were compared with the control group using two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone)

OGD Reduces the Levels of Intracellular Signaling Proteins Associated with Survival and Proliferation and Increases the Levels of Signaling Proteins Related with Death, While Atorvastatin Restores Normal Signaling Protein Levels

When evaluating the effects of OGD and atorvastatin on intracellular signals associated with proliferation of NSCs, the immunoreactivities of Ki67 and nestin, which reflect proliferative activity, were significantly decreased in NSCs by 8-h OGD but were increased by atorvastatin treatment in a concentration-dependent manner (Fig. 7a, b). Survival-related signals including PI3K, pAkt (Ser473), pGSK-3ß (Ser9), and Bcl-2 were also decreased in NSCs by 8-h OGD but were markedly increased with atorvastatin treatment in a concentration-dependent manner (Fig. 7a, b). In contrast, the expressions of Bax, cytosolic cytochrome *C*, cleaved caspase 9, and cleaved caspase 3, which are involved in cell death, were increased after exposure to 8-h OGD but were concentration-dependently decreased with



	All		Nestin		DCX		GFAP	
			Left	Right	Left	Right	Left	Right
	Count	% of All	% of All	% of All	% of All	% of All	% of All	% of All
Control	10,000	100.00%	49.83%	50.17%	49.43%	50.70%	49.34%	50.66%
8 hr OGD	10,000	100.00%	55.48%	44.52%	65.67%	34.35%	37.48%	62.52%
8 hr OGD + 0.01 μM Ator	10,000	100.00%	51.15%	48.85%	58.68%	47.32%	40.88%	59.12%
8 hr OGD + 0.1 μM Ator	10,000	100.00%	47.58%	52.42%	54.45%	45.72%	46.12%	53.88%
8 hr OGD + 1 μM Ator	10,000	100.00%	46.00%	54.00%	55.53%	44.53%	47.00%	53.00%
8 hr OGD + 10 μM Ator	10,000	100.00%	51.92%	48.08%	57.10%	42.90%	41.58%	58.42%



(N≥3)

Fig. 6 Effect of OGD and atorvastatin on the differentiation of NSCs. To confirm whether exposure to OGD and/or atorvastatin treatment affects the differentiation of NSCs, FACS analysis using antibodies for nestin, doublecortin (DCX—a neuronal marker), and GFAP (an astrocyte marker) was performed. The results show that exposure to 8-h OGD increases gliogenesis from NSCs, while atorvastatin significantly increases neurogenesis and inhibits gliogenesis. Data are presented as the mean (% of positive cells) ± SD of more than three independent experiments. Treatment groups were compared with the control group using two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone)

atorvastatin treatment (Fig. 7c, d). In terms of the ERK signaling pathway, which is another important signaling pathway for survival, 8-h OGD strongly reduced the expression of pERK (Th202/204) while increasing the expression of p-p38 (Th180/Tyr182) in NSCs. However, treatment with 0.1 and 1 μM atorvastatin restored these proteins to normal levels (Fig. 7e, f).

The PI3K and ERK Pathways Play Critical Roles in the Atorvastatin-Induced Rejuvenation of Stem Cell Characteristics of OGD-Injured NSCs

Because we found that OGD significantly affected the stem cell characteristics of NSCs, including survival, proliferation, and migration, while atorvastatin effectively restored these characteristics and revived the PI3K and ERK pathways in NSCs damaged by OGD, the roles of the PI3K and ERK pathways in the rejuvenation of NSCs by atorvastatin were investigated. Therefore, we pretreated NSCs with a PI3K or ERK inhibitor prior to OGD and atorvastatin treatment. Both PI3K and ERK inhibitors blocked the effect of atorvastatin on NSCs-damaged by OGD, so cell viability was decreased by approximately 20% compared with NSCs that were not pretreated with PI3K or ERK inhibitor (Fig. 8a, b, respectively).

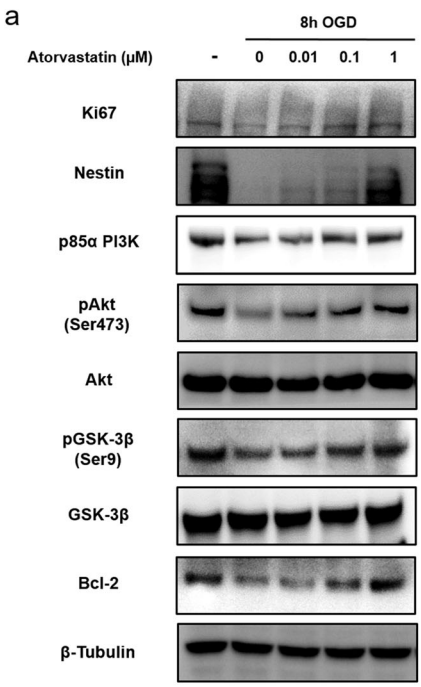
Discussion

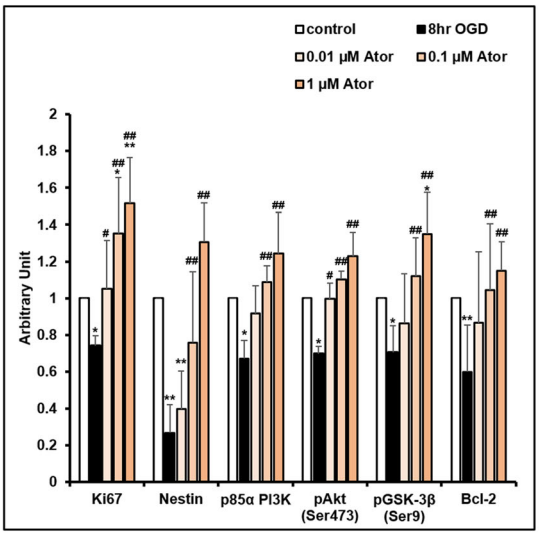
In the present study, we confirmed that oxygen–glucose deprivation (OGD), which is one of the most important pathogenic mechanisms of cerebral infarction [12, 13], causes severe damage to neural stem cells (NSCs) and changes their characteristics, including proliferation, migration, and differentiation,

which are critical factors for stem cells. OGD reduced the viability of NSCs in a time-dependent manner (Fig. 1b, c); significantly lowered the expression of Ki67, a proliferation marker, and nestin, an NSC marker (Fig. 4a); and markedly decreased the proliferation capacity of NSCs (Fig. 4b–e). OGD also strongly inhibited the migratory capacity of NSCs (Fig. 5) and induced glial differentiation of NSCs rather than neuronal differentiation (Fig. 6). Based on these findings, NSCs in the brain are likely to become damaged and lose their important characteristics when cerebral infarction occurs. These changes in NSCs prevent them from contributing to recovery after cerebral infarction. Due to the increase in the aging population, patients with cerebral infarction and its sequelae are rapidly increasing and have become major socio-economic burdens. However, there is not currently an established method to treat the sequelae of cerebral infarction. It has been suggested that one of the possible solutions to restore neurological function after cerebral infarction could be regeneration of the damaged brain by endogenous NSCs. However, as confirmed in the present study, endogenous NSCs are also damaged by ischemic stroke and can lose their critical stem cell characteristics so that they cannot aid in recovery of the injured brain. As a result, improving the function of NSCs could be helpful to patients recovering from brain damage after cerebral infarction. Recently, Sorrells et al. [13] proposed that neurogenesis in the dentate gyrus does not continue or is extremely rare in adult humans, raising questions about its contribution to brain repair or normal brain function. However, one important characteristic of adult neurogenesis by NSCs is its dynamic regulation by various physiological, pathological, and pharmacological stimuli, such as exercise, antidepressants, aging, epilepsy, and stroke [14]. In addition, Doetsch et al. [15] suggested that NSCs in the SVZ can be proliferated and differentiated to astrocytes after stroke and take part in glial scar formation, indicating that NSCs have different roles under normal and stressful conditions.

In the present study, atorvastatin rejuvenated NSCs injured by OGD and induced neuronal differentiation instead of astrocytic differentiation, suggesting the possibility of protecting NSCs and promoting neurogenesis after stressful brain damage. Atorvastatin has been suggested to have neuroprotective effects in many stressful conditions [4–6]. It has been also reported that atorvastatin has useful effects in ischemic stroke [5]. Considering the beneficial effects of atorvastatin on cerebral infarction, we hypothesized that atorvastatin could be involved in the rejuvenation of NSCs damaged by OGD, which has not yet been examined. To determine if atorvastatin rejuvenates NSCs after ischemic injury, we treated NSCs exposed to 8-h OGD with atorvastatin and evaluated viability, proliferation, migration, and differentiation in the NSCs. Atorvastatin effectively restored the viability of NSCs damaged by OGD (Figs. 2 and 3). In addition, atorvastatin significantly improved the proliferative capacity of OGD-damaged NSCs (Figs. 4 and 7). Additionally, the expression of Ki67 and nestin was significantly increased in atorvastatin-treated NSCs compared with NSCs only damaged by OGD (Figs. 4a, b and 7a, b). A CFU assay showed that proliferation of NSCs can be restored with atorvastatin treatment, even after OGD-induced injury (Fig. 4c–e). In terms of migratory capacity, atorvastatin improved the migratory capacity of NSCs injured by OGD (Fig. 5). OGD also induced glial differentiation of NSCs rather than neuronal differentiation (Fig. 6), which is compatible with previous findings [16]. Gliogenesis just after cerebral infarction is known to be beneficial, possibly because it prohibits the propagation of pathologic processes of cerebral infarction. However, gliogenesis can be harmful after the acute stage by forming glial scars that can inhibit neurogenesis and regeneration in the damaged brain; so many studies have aimed at reducing gliogenesis after the acute stage of cerebral infarction [16]. Considering that treatment with atorvastatin reverses this phenomenon and significantly increases neuronal differentiation and reduces glial differentiation (Fig. 6), atorvastatin may contribute to regeneration of the brain damaged by ischemic stroke by increasing neurogenesis and inhibiting gliogenesis. These findings suggest that atorvastatin can meaningfully contribute to the rejuvenation of NSCs damaged by OGD, resulting in regeneration of the brain by NSCs after cerebral infarction. However, in the present study, atorvastatin did not show a neuroprotective or rejuvenating effect at concentrations greater than 10 μM. Some studies have suggested that atorvastatin has anticancer effects through various mechanisms [17, 18], so it could be toxic to cells at high concentrations. Although there is no direct evidence that atorvastatin has toxicity on NSCs, Schulz el al. [19] demonstrated that atorvastatin at 10 μM and higher induces cell death in rat primary cortical neurons, in accordance with our previous data about rat motor neurons [4]. Therefore, it is possible that atorvastatin can be toxic to NSCs at high concentrations greater than 10 μM, as shown in the present study.

In terms of the protective effects of atorvastatin, diverse mechanisms have been proposed. Among the many important protective mechanisms of atorvastatin, the effect of atorvastatin on the phosphatidylinositol 3 kinase (PI3K) pathway is commonly accepted. The PI3K pathway is important in the survival, proliferation, and migration of stem cells. It has been reported that several neurotrophic factors can activate the PI3K pathway [20], which promotes the phosphorylation of Akt-1, leading to proliferation of NSCs [21]. Likewise, Takahashi et al. reported that the PI3K signaling pathway and its downstream protein Akt-1 in particular were of great importance in mouse embryonic stem cells (ESCs) and were considered crucial for unlimited survival and proliferation as well as in inhibition of apoptotic processes [22]. Hossini et al. reported that an inhibitor of the PI3K/Akt pathway dramatically and rapidly induced apoptosis in induced pluripotent stem cells (iPSCs) [23]. Moreover, Li et al. suggested that the PI3K/


a
8h OGD
Atorvastatin (μM) - 0 0.01 0.1 1
Ki67
Nestin
p85α PI3K
pAkt (Ser473)
Akt
pGSK-3β (Ser9)
GSK-3β
Bcl-2
β-Tubulin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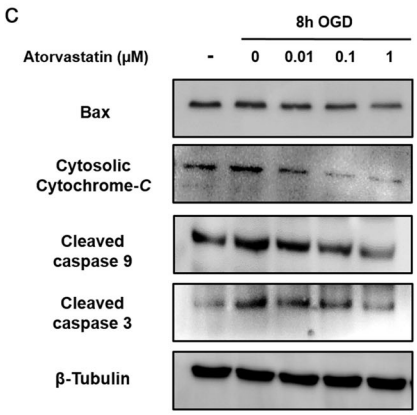
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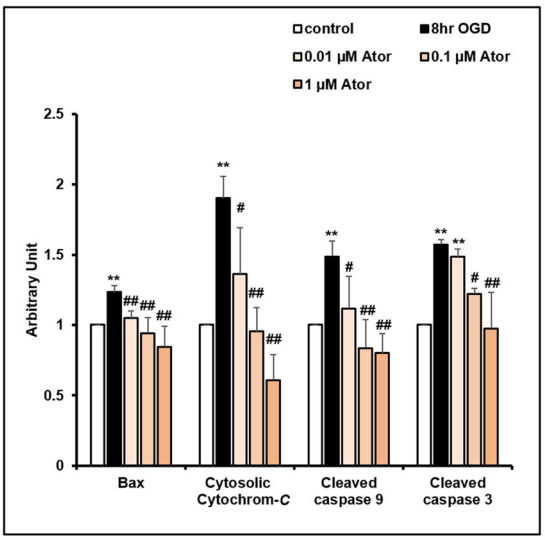
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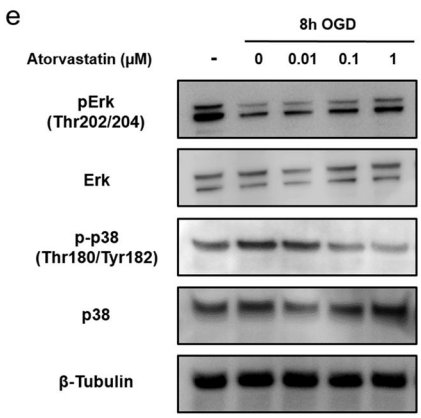
e
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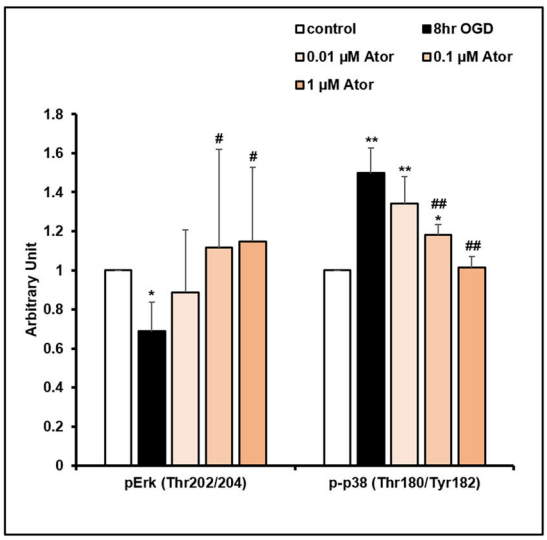
Erk
p-p38 (Thr180/Tyr182)
p38
β-Tubulin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control
8hr OGD
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0.1 μM Ator
1 μM Ator
Arbitrary Unit
pErk (Thr202/204)
p-p38 (Thr180/Tyr182)

◀ **Fig. 7** Effects of OGD and atorvastatin on intracellular signaling proteins associated with survival and proliferation. Using Western blotting, we demonstrated alterations in intracellular signaling proteins related to cell survival and proliferation after co-treatment with atorvastatin. Immunoreactivities (IRs) of Ki-67, nestin, PI3K, phosphorylated Akt (pAkt) (Ser473), Akt, phosphorylated GSK-3ß (pGSK-3ß) (Ser9), GSK-3ß, and Bcl-2, which are survival- and proliferation-related proteins, increased after co-treatment with 0.1 and 1 μM atorvastatin compared with NSCs that were treated with 8-h OGD alone (**a**, **b**). Bax, cytosolic cytochrome *C*, cleaved caspase 9, and cleaved caspase 3, which are cell death-related proteins, decreased after co-treatment with 0.1 and 1 μM atorvastatin compared with NSCs that were treated with 8-h OGD alone (**c**, **d**). Levels of pERK (Th202/204) increased and p-p38 (Th180/Tyr182) decreased in NSCs after co-treatment with 0.1 and 1 μM atorvastatin compared with NSCs that were treated with 8-h OGD alone (**e**, **f**). Data are expressed as percentages of the values in the control group and presented as the mean ± SD of five independent experiments. Treatment groups were compared with the control group using a one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone)

Akt signaling pathway activated by neuronal induction media enhances migration of neural-like cells toward stromal cell-derived factor-1alpha (SDF-1alpha) [24].

Another important mechanism is the effect of atorvastatin on the ERK pathway. The extracellular signal-regulated kinase 1/2 (ERK 1/2) pathway is associated with the cell cycle, transcription, and cellular proliferation [25]. Likewise, this pathway has been found to be important in stem cell survival and function. Li et al. [26] reported that caspase-3 activation was observed in the presence of a p-ERK 1/2-specific inhibitor and concluded that ERK 1/2 phosphorylation was a key event required for early neuronal differentiation and survival of ESCs. In a more recent study, Rhee et al. [27] showed that Raf-ERK signal activation in NSCs modulates neuronal differentiation and acquisition of cell proliferation.

It is well established that atorvastatin activates the PI3K pathway and gives rise to neuroprotection in many stressful conditions. Therefore, we examined whether atorvastatin also affected the PI3K pathway in regard to the rejuvenation of NSCs damaged by OGD. OGD for 8 h decreased the protein levels of p85a PI3K, phosphorylated Akt (Ser473), phosphorylated GSK-3β (Ser9), and Bcl-2, which are all associated with the PI3K pathway as well as survival and proliferation of NSCs (Fig. 7a, b). In contrast, OGD increased levels of Bax, cytosolic cytochrome-*C*, cleaved caspase 9, and cleaved caspase 3, which are directly related to cell death in NSCs (Fig. 7c, d). These alterations in the levels of intracellular signaling proteins by 8-h OGD are thought to be important in the loss of critical functions of NSCs. However, atorvastatin significantly increased the levels of survival-related proteins associated with the PI3K pathway and markedly reduced the levels of proteins related to cell death (Fig. 7a–d). Based on these findings, it is clear that OGD injures NSCs by inhibiting the PI3K pathway and atorvastatin can rejuvenate these cells by restoring PI3K signaling.

In terms of the ERK pathway, 8-h OGD slightly but significantly decreased the expression of ERK phosphorylated at Thr202/204, which is an activated form of ERK, while atorvastatin markedly increased this form of ERK above the normal level. Also, 8-h OGD markedly increased the level of phosphorylated p38 (Thr180/Tyr182), which is a well-known downstream signaling protein inhibited by ERK and positively associated with cell death in NSCs, and atorvastatin normalized this change (Fig. 7e, f). As discussed above, considering that the ERK pathway is very important in NSC survival and proliferation as well as in maintenance of stem cell characteristics, the effect of atorvastatin on the ERK pathway in NSCs damaged by OGD may contribute to the rejuvenation of NSCs in ischemic stroke.

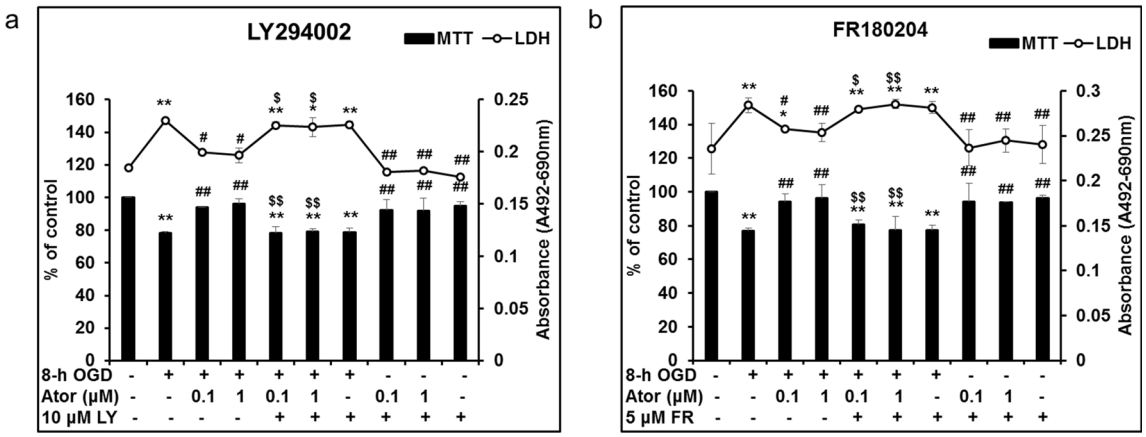

Fig. 8 Role of the PI3K and ERK pathways in the rejuvenation of OGD-injured NSCs by atorvastatin. Co-treatment with the inhibitors LY294002 (**a**) or FR180204 (**b**) almost completely blocks the protective effects of atorvastatin on the viability and cytotoxicity of OGD-treated NSCs. Data are presented as the mean (% of control) ± SD of 10 independent experiments. Treatment groups were compared with the control group using one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. the control group); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. the group that was treated with 8-h OGD alone); $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ (vs. the group that was treated with 8-h OGD and 0.1 or 1 μM atorvastatin)

To confirm the direct role of the PI3K and ERK pathways in the rejuvenation of OGD-damaged NSCs by atorvastatin, we pretreated NSCs with inhibitors of each pathway: LY294002, a direct Akt inhibitor, and FR180204, a direct ERK inhibitor. Treatment with these inhibitors almost completely blocked the beneficial effect of atorvastatin on the survival of NSCs (Fig. 8). These findings indicate that the effects of atorvastatin on OGD-damaged NSCs are directly associated with the PI3K and ERK pathways.

There are some limitations in this study. First, this study was performed using embryonic NSCs. However, because oxygen–glucose deprivation usually occurs in adult brain with ischemic stroke, it might be more appropriate to perform this study using adult NSCs. However, there are some hurdles to their use. Most adult NSCs have divide slowly, and their in vitro expansion might not be sufficient for preclinical studies [28]. In addition, primary embryonic and adult NSCs are known to have similar characteristics and can be maintained and differentiated in vitro as neurospheres without altering their functional properties over passaging [28, 29]. Thus, many previous studies have used embryonic NSCs instead of adult NSCs [10, 30]. Second, this study was performed in vitro, which can be very different from an in vivo environment. Therefore, we cannot confirm that OGD and atorvastatin will have the same effects on NSCs in vivo. However, as with most in vitro studies, we can show direct effects of OGD and atorvastatin on NSCs and confirm their mechanisms. Third, because we used an in vitro model of ischemic stroke, the treatment duration was relatively short when considering that atorvastatin is normally taken for a long time after ischemic stroke, so we could not examine the long-term effects of atorvastatin on NSCs after ischemic stroke. Further in vivo studies need to be performed to confirm the effect of atorvastatin on the rejuvenation of NSCs in the brain after cerebral infarction.

In conclusion, OGD significantly affected the characteristics of NSCs so that they could not contribute to recovery of the brain after cerebral infarction. However, treatment with atorvastatin just after cerebral infarction markedly restored the characteristics of NSCs including survival, proliferation, migration, and differentiation, and these revitalized NSCs could contribute to regeneration of the brain after cerebral infarction.

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Compliance with Ethical Standards

All animal procedures were conducted in accordance with Hanyang University's guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Hanyang University.

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