Atorvastatin Protects NSC-34 Motor Neurons Against Oxidative Stress by Activating PI3K, ERK and Free Radical Scavenging

Seok-Ho Lee · Na-Young Choi · Hyun-Jeung Yu · Jinse Park · Hojin Choi · Kyu-Yong Lee · Yong-Min Huh · Young Joo Lee · Seong-Ho Koh

Received: 20 September 2014 / Accepted: 30 November 2014 / Published online: 11 January 2015
© Springer Science+Business Media New York 2015

Abstract Although statins, or hydroxymethylglutaryl coenzyme A (HMG-Co A) reductase inhibitors, are generally used to decrease levels of circulating cholesterol, they have also been reported to have neuroprotective effects through various mechanisms. However, recent results have indicated that they may be harmful in patients with amyotrophic lateral sclerosis (ALS). In this study, we investigate whether atorvastatin protects motor neuron-like cells (NSC-34D) from oxidative stress. To evaluate the effects of atorvastatin or hydrogen peroxide or both on NSC-34D cells, the cells were treated with various combinations of these agents. To evaluate the viability of the cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and trypan blue staining were performed. Levels of free radicals and intracellular signaling proteins were evaluated using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and Western blotting, respectively. Atorvastatin protected NSC-34D cells against oxidative stress in a concentration-dependent manner. This neuroprotective effect of atorvastatin was blocked by LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor and by FR180204, a selective extracellular signal-related kinase (ERK) inhibitor. Atorvastatin treatment increased the expression levels of p85α PI3K, phosphorylated Akt, phosphorylated glycogen synthase kinase-3β, phosphorylated ERK, and Bcl-2, which are proteins related to survival. Furthermore, atorvastatin decreased the levels of cytosolic cytochrome C, Bax, cleaved caspase-9, and cleaved caspase-3, which are associated with death in oxidative stress-injured NSC-34D cells. We conclude that atorvastatin has a protective effect against oxidative stress in motor neurons by activating the PI3K and ERK pathways as well as by scavenging free radicals. These findings indicate that statins could help protect motor neurons from oxidative stress.

Keywords Statin · Amyotrophic lateral sclerosis · Phosphatidylinositol 3-kinase · Extracellular signal-related kinase

Introduction

Statins (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors) are potent cholesterol-lowering drugs used to treat hypercholesterolemia [1]. Statins retard atherosclerotic plaque development, stabilizes existing plaque progression, and may reduce cardiovascular and cerebrovascular mortality and morbidity [2]. In addition, they have been reported to help prevent or treat certain neurodegenerative diseases such as Parkinson’s disease [3], Alzheimer’s disease [4], multiple sclerosis [5], and traumatic brain injury [6].
However, in 2007, the WHO Foundation Collaboration Centre for International Drug Monitoring reported an association between statin use and an amyotrophic lateral sclerosis (ALS)-like syndrome [7]. Following the 2007 WHO study, Colman et al. [8] reported a similar result based on data from the US Food and Drug Administration’s spontaneous event reporting system [8]. Since then, the association between statins and ALS has become controversial. A recent study reported a strong association between statin medications and more rapid ALS progression, although the study was performed in only a small number of patients with the disease [9]. In contrast, a retrospective investigation of a large number of patients with ALS did not find any influence of statins on survival, concluding that statin treatments are safe for patients with ALS [10]. A recent in vivo study reported that statin treatment delayed symptomatic and neuropathological deterioration in wobbler mice, an animal model of human motor neuron disease [11]. Until now, no pathogenic mechanism has been proposed to explain the link between statins and ALS.

NSC-34 cells are hybrid cells derived from the fusion of mouse neuroblastomas and motor neurons from embryonic mouse spinal cords [12]. These cells have been widely used as in vitro models of ALS and other disorders affecting motor neurons. They are used especially after inducing differentiation by serum depletion [13], because primary motor neurons have a limited proliferative capacity and are short-lived in culture. In addition, they are potentially useful for studying motor neuron dysfunction in response to toxins [14]. Oxidative stress has been emphasized as a common pathogenic mechanism in neuronal cell death; it thus plays an important role in several neurological disorders [15, 16]. It has also been implicated as a pathogenic mechanism in motor neuron diseases [17]. Considering that statins have antioxidant effects, it seemed possible that they would prevent motor neuron death caused by oxidative stress. We have therefore investigated whether atorvastatin protects NSC-34 cells from oxidative stress and have examined its mechanisms of action.

Materials and Methods

Materials

Atorvastatin was a gift from Chong Kun Dang Co., Ltd. (South Korea). Before use, it was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 % (v/v), and then further diluted in culture medium to the desired final concentration. LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor and FR180204, a selective extracellular signal-related kinase (ERK) inhibitor, were purchased from Sigma (Saint Louis, MO, USA) and Calbiochem (Billerica, MA, USA), respectively.

Primary Culture and Treatment of NSC-34 Cells

The neuroblastoma-spinal motor neuron fusion cell line, NSC-34, was purchased from CELLutions (CELLutions Biosystems Inc., Ontario, Canada).

NSC-34 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin streptomycin (PS) [12] and were subcultured every 3–5 days. To slow cell proliferation and enhance differentiation, the medium was exchanged for 1:1 DMEM and Ham’s 12 with 1 % FBS, 1 % PS, and 1 % non-essential amino acids [13]. The medium was changed every 2 days and the cells were grown for up to 7 days to allow them to differentiate into motor neuron-like cells with increased neurite formation [13].

To test whether the differentiated NSC-34 cells expressed motor neuronal markers, we performed immunostaining for choline acetyltransferase (ChAT). The cells were grown on poly-L-lysine-coated cover slips (BD Biosciences, USA). After fixation for 15 min at room temperature within a solution containing 4 % paraformaldehyde in phosphate-buffered saline (PBS), they were washed three times with 2 mL of PBS and then permeabilized with 0.5 % Triton X-100 in PBS for 5 min at room temperature. After three washes with PBS, endogenous peroxidase was quenched with 3 % H2O2 for 20 min at room temperature followed by three more washes with PBS. The cells were then incubated with blocking buffer (5 % fetal bovine serum in PBS) for 1 h at room temperature. They were subsequently incubated overnight at 4 °C with anti-ChAT antibody (Millipore, USA) diluted 1:250 in 2 % fetal bovine serum in PBS. After washing three times with PBS, the cells were incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) antibody (Life Technologies, US) for 1 h at room temperature diluted 1:100, washed three times with PBS, mounted with a drop of mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) (ProLong Gold antifade reagent with DAPI, Invitrogen, USA) and examined by fluorescence microscopy.

To determine the best oxidative stress conditions for our experiments, several H2O2 concentrations were tested. After 24 h of H2O2 treatment, the plates were washed and cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue staining [18].

H2O2 levels were evaluated using a luminol-dependent chemiluminescence assay [19]. Using HPLC [20], we indirectly assessed the reactive hydroxyl radical (OH ) levels produced by the H2O2 by quantifying the 2,5- and 2,3-dihydroxybenzoic acids (DHBA) generated by salicylate hydroxylation (Wako Pure Chemical Industries Ltd., Osaka, Japan).
Japan), using 2,5- and 2,3-DHBA standards obtained from Sigma. After washing several times, the H$_2$O$_2$ and reactive OH$^-$ levels in the neurons treated with 100 μM H$_2$O$_2$ alone were not significantly different from those first treated with atorvastatin. Cells harvested after 24 h of H$_2$O$_2$ treatment were used immediately for immunodetection of PI3K/Akt, GSK-3β, ERK, Bcl-2, Bax, cytoplasmic cytochrome C, cleaved caspase-9 and cleaved caspase-3 [18].

Finally, to examine the role of the PI3K and ERK pathways in atorvastatin neuroprotection, we treated NSC-34D cells with the following combinations: 5 μM LY294002 a PI3K inhibitor and 5 μM FR180204 a selective ERK inhibitor, with/without atorvastatin and with/without H$_2$O$_2$ for 24 h. NSC-34D cells were divided into nine groups: control (group 1), 100 μM H$_2$O$_2$ (group 2), 100 μM H$_2$O$_2$+1 μM atorvastatin (group 3), 100 μM H$_2$O$_2$+1 μM atorvastatin+5 μM LY294002 (group 4), 100 μM H$_2$O$_2$+1 μM atorvastatin+5 μM FR180204 (group 5), 100 μM H$_2$O$_2$+5 μM LY294002 (group 6), 100 μM H$_2$O$_2$+5 μM FR180204 (group 7), 5 μM LY294002 (group 8), and 5 μM FR180204 (group 9). Cell viability was assessed using the LDH and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-}

**Determinition of Free Radical Production**

To measure free radical production induced by H$_2$O$_2$, NSC-34D cells were treated with 0 (control) or 100 μM H$_2$O$_2$ for 1 h, washed three times with DMEM-free medium, and then incubated for 15 min with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR, USA). To evaluate the effect of atorvastatin on free radical production by H$_2$O$_2$, cells were simultaneously treated with 0.1, 1, or 10 μM atorvastatin. DCFH-DA freely crosses cell membranes and is hydrolyzed by cellular esterases to 2′,7′-dichlorodihydrofluorescein (DFCH2), which is not fluorescent, but is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of peroxides. Thus, DCF fluorescence indicates intracellular hydrogen peroxide levels, but not superoxide levels. DCF accumulation can be measured as an increase in fluorescence at 525 nm when excited at 488 nm and was measured using a microplate fluorescence reader (FL600; D.I. Biotech Ltd., Seoul, Korea) [18].

**Western Blotting**

For Western blot analysis, aliquots of $5 \times 10^6$ cells were briefly washed twice in cold PBS and incubated for 10 min on ice in a lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02 % sodium azide, 0.2 % SDS, 100 μg/mL phenylmethylsulfonylfluoride (PMSF), 50 μL/ml aprotinin, 1 % octylphenoxypolyethoxyethanol 630, 100 mM NaF, 0.5 % sodium deoxycholate, 0.5 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA)]. The lysates were centrifuged at 10,000 g and levels of p85α PI3K, pAkt, pGSK-3β, pERK, Bcl-2, Bax, cleaved caspase-9, and cleaved caspase-3 in each cell lysate were then evaluated. To measure released cytoplasmic cytochrome C, cells were suspended in sucrose-supplemented cell extraction buffer (SCEB: 300 mM sucrose, 10 mM HEPES at pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM MgCl$_2$, 1 mM DTT, 10 μM cytochalasin B, and 1 mM PMSF) after washing, left on ice for 30 min, and homogenized with 50 strokes in an ice-cold Dounce homogenizer. Unbroken cells and nuclei were pelleted by centrifugation for 10 min at 2000 g. Mitochondria were collected by centrifuging the resulting supernatant at 13,000 g for 10 min. The supernatant (post-mitochondrial fraction) was immunoblotted for cytochrome C. Protein concentrations were determined with a Bio-Rad protein assay kit (Hercules, CA, USA). Samples containing equal amounts (20 μg) of protein were resolved by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
(SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 2% skimmed milk and incubated with primary antibodies, i.e., antibodies against p85α PI3K (1:1000, Cell signaling, Beverly, MA, USA), pAkt (Ser473; 1:100, Cell Signaling), pGSK-3β (Ser9; 1:100, Cell signaling), pERK (Thr202/204; 1:500, Cell Signaling), Bcl-2 (1:1000, Cell Signaling), Bax (1:500, Cell Signaling), cytochrome C (1:200, Cell signaling), cleaved caspase-9 (1:500, Cell Signaling) and cleaved caspase-3 (Asp 175; 1:500, Cell Signaling). The membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST) and then processed using an HRP-conjugated anti-rabbit or anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) followed by enhanced chemiluminescence (ECL) detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA) [18]. The results were quantified with an image analyzer (Bio-Rad, Quantity One-4,2,0).

Statistical Analysis

All data are presented as mean±SEM of five or more independent experiments. To test for a normal distribution, the Kolmogorov–Smirnov test was performed on all data sets. Statistical comparisons between groups were done using t tests and comparisons between three or more groups were performed by one-way ANOVA followed by Tukey’s post hoc comparisons. Two-tailed p values less than 0.05 were considered statistically significant. All statistical analyses were performed with SPSS 17.0 software for Windows (SPSS, Chicago, IL, USA).

Results

Effect of Atorvastatin Alone on the Viability of NSC-34 Cells

We confirmed the production of differentiated NSC-34 (NSC-34D) cells by immunocytochemical staining for choline acetyltransferase, a motor neuron marker (Fig.1). To assess the effect of atorvastatin on NSC-34D cell viability, we treated NSC-34D cells with several atorvastatin concentrations for 24 h and measured cell viability by MTT assay. There was no significant reduction in cell viability following atorvastatin treatment up to a concentration of 1 μM. However, a solution concentration of 10 μM atorvastatin slightly decreased cell viability (Fig. 2a).

Effect of Atorvastatin on NSC-34 Cells Injured by Oxidative Stress

To evaluate NSC-34D cell viability after oxidative stress, NSC-34D cells were treated with different H2O2 concentrations for 24 h and cell viability was measured using the MTT assay. As shown in Fig. 1b, H2O2 significantly reduced NSC-34D viability in a dose-dependent manner. An optimal concentration of 100 μM H2O2 was considered because it produced a cell viability of 60–70%; therefore, this concentration was selected for use in subsequent experiments (Fig. 2b).

To evaluate the ability of atorvastatin to protect against oxidative stress, NSC-34D cells were simultaneously treated with atorvastatin (0.1, 1, and 10 μM) and 100 μM H2O2 for 24 h. Atorvastatin significantly rescued cell viability up to 1 μM (Fig. 2c). TUNEL and DAPI staining revealed that...
100 μM H₂O₂ increased apoptosis, and this was significantly reduced by combining H₂O₂ with 1 μM atorvastatin (Fig. 3).

Antioxidant Effects of Atorvastatin

To better understand free radical production induced by oxidative stress in NSC-34D cells, free radical levels in NSC-34D cells treated with 100 μM H₂O₂ for 1 h were measured using DCFH-DA (Fig. 4). Cells treated with 100 μM H₂O₂ produced significantly more free radicals than the control group (p<0.01). To examine any free radical scavenging effect of atorvastatin, NSC-34D cells were treated with H₂O₂ and 0.1, 1, or 10 μM atorvastatin for 1 h. The production of free radicals due to oxidative stress was reduced in a dose-dependent manner by atorvastatin up to a 1 μM concentration (p<0.01).

Effects of Atorvastatin on Intracellular Signaling Proteins

Homogenates of NSC-34D cells were analyzed by immunoblotting to determine the effect of atorvastatin on p85α PI3K, pAkt, pGSK-3β, pERK, and Bcl-2, all of which are involved in neuron survival. The expression of p85α PI3K, pAkt, pGSK-3β, pERK, and Bcl-2 increased in NSC-34D cells treated with 100 μM H₂O₂ and 1 μM atorvastatin, compared to cells treated with H₂O₂ alone (p<0.05, Fig. 5a–e).

Role of the PI3K and ERK Pathways in the Neuroprotective Effects of Atorvastatin

To evaluate the roles of the PI3K and ERK pathways on the neuroprotective effects of atorvastatin, we used LY294002, a PI3K inhibitor and FR180204, a selective ERK inhibitor.
Cells were separated into nine groups and each was treated for 24 h: control (group 1), 100 μM H₂O₂ (group 2), 100 μM H₂O₂+1 μM atorvastatin (group 3), 100 μM H₂O₂+1 μM atorvastatin+5 μM LY294002 (group 4), 100 μM H₂O₂+1 μM atorvastatin+5 μM FR180204 (group 5), 100 μM H₂O₂+5 μM LY294002 (group 6), 100 μM H₂O₂+5 μM FR180204 (group 7), 5 μM LY294002 (group 8), and 5 μM FR180204 (group 9). Pretreatment with the PI3K inhibitor in group 4 reduced cell viability by approximately 14 % compared with group 3 (100 μM H₂O₂+1 μM atorvastatin) (Fig. 6). In addition, pretreatment with the selective ERK inhibitor (group 5) reduced viability by about 10 % compared with group 3 (Fig. 6). However, pretreatment with the PI3K inhibitor (group 8) and the ERK inhibitor (group 9) did not affect NSC-34D cell viability under oxidative stress (Fig. 6).

**Discussion**

In the present study, atorvastatin did not affect NSC-34D cell viability at concentrations up to 1 μM (Fig. 2a). However, H₂O₂ significantly decreased cell viability in a dose-dependent manner (Fig. 2b), and the addition of atorvastatin markedly reduced H₂O₂-induced neuronal cell death (Figs. 2c and 3). We also found that it markedly reduced free radical production due to oxidative stress (Fig. 4). Furthermore, oxidative stress decreased levels of survival signaling molecules such p85α PI3K, pAkt, pGSK-3β, pERK, and Bcl-2 and increased levels of death signaling molecules, including Bax, cytosolic cytochrome C, cleaved caspase-9, and cleaved caspase-3 (Fig. 5), and co-treatment with H₂O₂ and atorvastatin significantly increased these survival signals and decreased death signals (Fig. 5). We also observed that the neuroprotective effects of atorvastatin were associated with PI3K and ERK activation because PI3K and ERK inhibitors antagonized its neuroprotective effects (Fig. 6). All these findings suggest that atorvastatin protects NSC-34D cells from oxidative stress by activating the PI3K and ERK pathways, as well as having a direct antioxidant effect.

Statins are known to have diverse neuroprotective effects through various mechanisms [21, 22]. The best characterized neuroprotective mechanisms involve statin-induced activation of neuroprotective signaling pathways, including the PI3K signaling pathway [21–23] and the Ras-ERK-signaling pathways [24, 25], as well as direct antioxidant effects [26], and
inhibition of the interaction between leukocyte function antigen-1 and intercellular adhesion molecule-1 [27]). Furthermore, statins have been proposed as potential treatments for diverse diseases such as certain types of cancer and neurodegenerative disease [28]. Paradoxically, it has been suggested, in the case of cancer, that statins increase apoptosis and alter levels of Bcl-2 family members [29, 30] whereas studies mainly using noncancerous cells report the opposite effects [31, 32].

The PI3K and ERK pathways, which are essential for cell survival, are activated by phosphorylation [19, 20, 33]. In the nervous system, PI3K pathway activation is involved in cell survival and cell proliferation in response to growth factors and cytokines [15, 16, 19]. Activated PI3K phosphorylates and activates Akt. pAkt remains in the cytosol where it phosphorylates and inhibits several cytosolic pro-apoptotic substrates. pAkt can also move to the nucleus and regulate some transcriptional factors [15, 16]. The ERK pathway also plays an important role in neuronal cell survival [33, 34]. In neurons, ERK is mainly activated by growth factors and is associated with cell proliferation, differentiation, and development [33, 34]. Such actions are best examined in the context of growth factor signaling via receptor tyrosine kinases [33, 34]. Receptor tyrosine kinases activate downstream targets such as small G proteins, Ras, Raf-1, and mitogen-activated protein kinase kinase (MEK). ERK activation by MEK is involved in neuronal survival and plays various roles in the activity-dependent regulation of neuronal function [33, 35]. There have been no reports of the effects of atorvastatin on the PI3K and ERK pathways in motor neurons injured by oxidative stress.

Therefore, we investigated whether the effects of atorvastatin on the PI3K and ERK pathways have a significant role in protecting NSC-34D cells under oxidative stress. Western blotting demonstrated that atorvastatin affects the PI3K and ERK pathways in the NSC-34D cells that are injured by oxidative stress. Thus levels of p85αPI3K, pAkt, pGSK-3β, pERK, and Bcl-2 increased in NSC-34D cells under oxidative stress when they were treated with 1 μM atorvastatin, suggesting that the latter activates the PI3K and ERK pathways in cells injured by oxidative stress.

To confirm whether the PI3K and ERK pathways are directly involved and important in the neuroprotection afforded by atorvastatin, LY294002 a PI3K inhibitor, and FR180204 a selective ERK inhibitor, were applied to NSC-34D cells treated with H2O2 and atorvastatin. These inhibitors blocked the neuroprotective effects of atorvastatin. Additionally, in order to investigate the effect of both inhibitors on NSC-34D cells under oxidative stress, NSC-34D cells were treated with LY294002+H2O2, FR180204+H2O2, LY294002
Role of the PI3K and ERK pathways in the neuroprotective effects of atorvastatin on NSC-34D motor neuron-like cells injured by oxidative stress. To the best of our knowledge, this is the first report that shows PI3K and ERK pathway activation plays a critical role in the neuroprotective effects of atorvastatin. To confirm these findings indicate that atorvastatin directly activates the PI3K and ERK pathways and that this activation plays an important role in the neuroprotective effects of atorvastatin. To the best of our knowledge, this is the first report that shows that PI3K and ERK pathway activation plays a critical role in the neuroprotective effects of atorvastatin on NSC-34D motor neuron-like cells injured by oxidative stress.

Oxidative stress is important in the pathologic mechanisms of several neurological diseases, including ALS. Both sporadic and familial ALS involve pathological SOD1 [36, 37], a major source of H2O2 [38, 39]. H2O2 can act as a second messenger in signaling processes and can produce hydroxyl radicals and oxidize thiols and cysteines [40]. As H2O2 levels increase, thiols are reduced and thiolate-dependent enzymes are inhibited, leading to oxidative damage to lipids, proteins, and DNA. Furthermore, superoxide and NO released from activated microglia may act as exogenous toxins to motor neurons [41]. Oxidative stress probably accelerates disease progression.

Thus, many researchers have sought to reduce oxidative stress-induced neuronal cell death. Antioxidants have been reported to protect neurons from oxidative stress in several in vitro models of neurodegenerative diseases, including ALS [38]. Considering that oxidative stress plays an important role in ALS, it is essential to determine if the protective effects of atorvastatin in ALS are due to an antioxidant effect [38]. In our study, atorvastatin reduced free radical production induced by H2O2 in NSC-34D cells. This antioxidant effect may well contribute to the neuroprotective effects of atorvastatin.

However, we also demonstrated that the neuroprotective effect of atorvastatin was exerted under certain concentrations. Specifically, atorvastatin protected NSC-34D cells against oxidative stress-induced cell death when administered at concentrations in the high nanomolar to low micromolar (1 μM) range. However, this protective effect disappeared at an atorvastatin concentration of 10 μM, at which NSC-34D cell death was induced. This toxic effect of atorvastatin might be due to the high concentration at 10 μM; this finding is similar to two previous reports that showed that high micromolar concentrations of statins exerted pro-apoptotic effects in rat cortical neurons and neuroblasts [42, 43]. On the other hand, considering that such high micromolar concentrations cannot be attained in patients who are taking the recommended daily doses of atorvastatin, we can conclude that atorvastatin has neuroprotective effects within the appropriate concentrations that are reached in clinical practice.

There are some limitations to this study. The micromolar doses of atorvastatin that were used are not physiologically measured. LY294002 and FR180204 blocked the neuroprotective effects of atorvastatin. However, neither inhibitor used alone affected the viability of NSC-34D cells under oxidative stress. The data of five independent experiments is presented as mean (percent of control)±SD. The treatment groups were compared using Tukey’s test after one-way ANOVA. *p<0.05 and **p<0.01 compared with the control group, $\text{p}<0.05$ and $\text{p}<0.01$ (compared with cells treated with H2O2 alone).
relevant. Because this was an in vitro study to elucidate the neuroprotective effects of atorvastatin, long-term treatment with atorvastatin at lower concentrations was not feasible. Therefore, atorvastatin was used at higher concentrations for a relatively short duration of 24 h.

We did not determine the precise point of action of atorvastatin. Although this study showed that PI3K and ERK activation is important in the neuroprotective mechanism of atorvastatin, this activation might be due to an indirect effect, such as a nonspecific increase of the rate of phosphorylation reactions. The exact mechanism of action of atorvastatin needs to be further studies.

In conclusion, despite the limitations of this study, our results suggest that atorvastatin can prevent oxidative stress-induced neurotoxicity in motor neurons and that the neuroprotective effects of atorvastatin against oxidative stress-induced neurotoxicity are linked to PI3K and ERK activation, as well as to the inherent antioxidant effects of atorvastatin. These findings correlate with results from previous clinical trials of statins in patients with ALS. Our results suggest that statin therapy has neuroprotective effects in patients with ALS.

Acknowledgments This work was supported by a grant from the Korea Research Foundation (2012R1A1B3000473) and a grant from the NanoBio R&D Program of the Korea Science and Engineering Research Foundation (2012R1A1B3000473) and a grant from the Ministry of Education, Science and Technology (2007–04717).

Conflict of Interest None.

References


@ Springer
doi:10.1016/j.tips.2007.05.001
Improvement of nitric oxide-dependent vasodilatation by HMG-
CoA reductase inhibitors through attenuation of endothelial superox-
27. Wetz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J,
inhibit leukocyte function antigen-1 by binding to a novel regulatory
28. Wood WG, Igbavboa U, Muller WE, Eckert GP (2013) Statins, Bcl-
2, and apoptosis: cell death or cell protection? Mol Neurobiol 48(2):
29. Spamanato C, De Maria S, Sarnataro M, Giordano E, Zanfardino M,
cell growth by inducing apoptosis correlated to activation of Bax and
941. doi:10.3892/ijo.2011.1273
351–357. doi:10.3727/096504013X13657689382897
31. Johnson-Anuna LN, Eckert GP, Franke C, Igbavboa U, Muller WE,
Wood WG (2007) Simvastatin protects neurons from cytotoxicity by
doi:10.1111/j.1471-4159.2006.04375.x
32. Franke C, Noldner M, Abdel-Kader R, Johnson-Anuna LN,
Gibson Wood W, Muller WE, Eckert GP (2007) Bcl-2 upregu-
lation and neuroprotection in guinea pig brain following chronic
j.nbd.2006.10.004
kinase signalling in neurons. Curr Opin Neurobiol 9(5):544–553. doi:
10.1016/S0959-4388(99)00010-0
phosphatidylinositol 3-kinase and extracellular signal-regulated ki-

nase pathways in the neuroprotective effects of cilnidipine against
hypoxia in a primary culture of cortical neurons. Neurochem Int
61(7):1172–1182. doi:10.1016/j.neuint.2012.08.010
35. Hetman M, Xia Z (2000) Signaling pathways mediating anti-
531–545
147–159. doi:10.1146/annurev.bi.44.070175.001051
nuclear aggregates of superoxide dismutase-1 are regularly present in
patients with amyotrophic lateral sclerosis. Acta Neuropathol 121(5):
mechanism of neurodegeneration and a therapeutic target. Biochem
03.008
b09020378
41. Li Q, Spencer NY, Pantazis NJ, Engelhardt JF (2011) Alsin and
SOD1(G93A) proteins regulate endosomal reactive oxygen species
production by glial cells and proinflammatory pathways responsible
jb.M111.279711
42. Tanaka T, Tatsuno I, Uchida D, Moroo I, Morio H, Nakamura S,
Geranylgeranylpyrophosphate, an isoprenoid of mevalonate cas-
cade, is a critical compound for rat primary cultured cortical neurons
to protect the cell death induced by 3-hydroxy-3-methylglutaryl-CoA
(2001) Lovastatin induces apoptosis of spontaneously immortalized
rat brain neuroblasts: involvement of nonsterol isoprenoid biosyn-