CHOLESTEROL-INDEPENDENT, MAPK/ERK SIGNAL-MEDIATED SIMVASTATIN POTENTIATION OF NERVE GROWTH FACTOR-INDUCED NEURITE OUTGROWTH IN PC-12 CELLS

Takashi Ochiai[a], *Kanemasa Fukuda*[a], *Masakazu Kasuya*[a], *Akira Sakata*[a], *Hiroshi Shimeno*[a], *Akihisa Toda*[b], *Reiko Eyanagi*[b], *Shinji Soeda*[a,*]

[a]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan
[b]Department of Hygienic Chemistry, Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan

*Corresponding author. Fax: +81-92-863-0389. E-mail: ssoeda@fukuoka-u.ac.jp

Abstract

Statins are potent cholesterol-lowering drugs. Some lipophilic derivatives of the statins are reported to show both neurotoxic and neuroprotective effects. However, the molecular mechanisms by which the statins affect the survival of neurons are not fully understood. In this study, we examined the effects of simvastatin, lovastatin and mevastatin on the neuronal differentiation of rat pheochromocytoma (PC-12) cells. Exposure of undifferentiated PC-12 cells to each statin alone at concentrations of ≥10 μM for ~48 h reduced the cells' survival, while exposure to concentrations of ≤1 μM did not. Treatment with 1 μM lovastatin or simvastatin induced a transient neurite outgrowth. In combination treatments for 72 h with nerve growth factor (NGF), simvastatin promoted NGF-induced neurite outgrowth more potently than an additive effect. We found that simvastatin most effectively promotes the phosphorylation of extracellular signal-regulated kinase (ERK), but not of protein kinase B (Akt), and contributes to prolonging the NGF-induced phosphorylated state of ERK at higher levels than those caused by NGF alone. Simvastatin decreases synthesis of cholesterol and its precursor, farnesylpyrophosphate, which is essential for the biological activity of Ras. However, treatment with 1 μM simvastatin alone did not affect the cholesterol content and Ras activity. Our results suggest that simvastatin at limited low concentrations is not merely an inhibitor of Ras/Raf/ERK pathway but rather a potent activator of MAPK/ERK pathway, which plays a key role in the neuronal differentiation.

Introduction

Statins, the inhibitors of HMG-CoA reductase, are important drugs in the treatment of hypercholesterolemia (Veillard and Mach, 2002). Retrospective case-control studies reported that the use of statins potentially reduces the development of Alzheimer's disease and vascular dementia (Jick, Zornberg *et al.,* 2000; Wolozin, Kellman *et al.,* 2000), suggesting the drugs have a broad neuroprotective effect. It is also reported that

treatment with some statins produces side effects on the central nervous system, such as sleep disturbance (Sinzinger, Mayr *et al.,* 1994), and an increased risk of neuropathy (Gaist, Jeppesen *et al.,* 2002; Jeppesen, Gaist *et al.,* 1999).

Simvastatin and lovastatin are inactive lactone prodrugs. Because they are highly lipophilic, these statins can permeate the blood-brain barrier (Saheki, Terasaki *et al.,* 1994). In PC-12 cells, simvastatin inhibits cell division and induces neurite outgrowth (Sato-Suzuki and Murota, 1996). The statin also induces a transient activation of high affinity NGF receptor, TrkA, and morphological differentiation with fatal outcome (Kumano, Mutoh *et al.,* 2000). However, in HT22 cells, a murine hippocampal cell line, lovastatin and mevastatin potentiate caspase-3 activity when apoptotic signal transduction is initiated by staurosporine (Föcking, Besselmann et al., 2004). In this study, to clarify the conflicting roles of these statins in neurons, we examined their effects on the NGF-induced differentiation of PC-12 cells over a wide range of concentrations. Low concentrations of lovastatin and simvastatin promoted neurite outgrowth by activating the mitogen-activated protein kinase (MAPK)/ERK pathway, while high concentrations of the statins induced cell death. Simvastatin had the greatest effect on neuroplastic potency; lovastatin had the next greatest effect and mevastatin had the smallest. In addition, simvastatin at low concentrations was the most effective at maintaining NGF-induced phosphorylated state of ERK without alteration in cholesterol content and activated Ras in the cells. We report first evidence that simvastatin at concentrations ineffective in cholesterol synthesis aids NGF-related nerve differentiation and survival by enhancing intracellular signaling through the MAPK/ERK pathway.

Materials and Methods

Materials

The following materials were obtained commercially: 2.5 S NGF from CHEMICON International, Temecula, CA; mevastatin, lovastatin, simvastatin, PD98059 and LY294002 from CALBIOCHEM, La Jolla, CA; Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum from GIBCO BRL Life Technologies, Inc.

Cell cultures

The rat PC-12 cell line was obtained from the Dai-Nippon Seiyaku Co., Osaka,

Japan. Stock cultures of undifferentiated PC-12 cells were maintained in DMEM supplemented with 10 % FBS, 5 % inactivated horse serum, 50 units/ml penicillin and 100 μg/ml streptomycin at 37℃ in a humidified 5 % CO_2 atmosphere. Prior to experiments, the growing cells were seeded in collagen-coated 6-well plates (2.5×10^4 cells/well).

Measurement of cell viability

PC-12 cells in 100 μl DMEM supplemented with 10 % FBS, 5 % inactivated horse serum, 50 units/ml penicillin and 100 μg/ml streptomycin were seeded in each well of 96-well plates (5×10^3 cell/well). Incubation followed at 37℃ for 24 h. After being washed with DMEM, the cell layers were incubated in DMEM containing 1% FBS alone or with 0.1-50 μM mevastatin, lovastatin or simvastatin at 37℃ for set periods ranging from 0 h to 48 h. Cell viability was assayed with a Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) to count living cells by combining 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium (WST-8) and 1-methoxyphenazine methosulfate (1-methoxy-PMS).

Measurement of neurite outgrowth

PC-12 cells were cultured at a sufficiently low cell density to measure neurites that do not contact neurites from other cells. After being washed with DMEM, the cell layers were incubated for 24 or 72 h in DMEM containing 1% FBS with 50 ng/ml NGF alone, 1 μM of each statin or NGF plus a statin. Three different phase-contact microscopic fields (×200) per well were photographed. The total length of neurites on 30 cells in each photograph was scanned and quantified using an NIH Image software. The neurites were restricted to those of the cells that are not associated in clusters.

Western blot analyses of phosphorylated ERKs and Akt

Confluent cultures of undifferentiated PC-12 cells in collagen-coated 75 cm^2 culture flasks were incubated at 37℃ for set periods ranging from 0 h to 48 h in DMEM containing 1% FBS with 50 ng/ml NGF alone, 1 μM of each statin or a combination of NGF and a statin. The cell layers were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate and harvested. The cells were pelleted by centrifugation, and extraction followed for 10 min in 100 μl of a lysis buffer consisting of 10 mM HEPES buffer (pH 7.5); 50 mM KCl; 3 mM magnesium acetate; 0.3 mM EDTA; 10 % glycerol; 0.5 % Nonidet P-40; leupeptin (10 μg/ml); and antipain (10 μg/ml). After centrifugation at 12,000 × g for 15 min at 4℃, the resulting supernatants were tested for the presence of phosphorylated ERKs or Akt. Protein concentrations were measured with a DC Protein Assay kit (Bio-Rad). Equal amounts of the proteins (20 μg each) were separated by 10 % SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membrane (BIO RAD). The transferred proteins were reacted with rabbit polyclonal antibodies against phosphorylated ERK 1/2 or with rabbit polyclonal antibodies against phosphorylated or non-phosphorylated Akts 1/2/3 from Santa Cruz Biotechnology, Inc. The resulting immunocomplex was further reacted with peroxidase-conjugated secondary antibodies and made visible with 4-chloro-1-naphthol as the peroxidase substrate.

Measurement of Ras activation

Confluent cultures of PC-12 cells in 75 cm^2 culture flasks were incubated in DMEM containing 1% FBS with NGF (50 ng/ml) alone, simvastatin (1 μM) alone or NGF plus simvastatin for the definite times. Active Ras in the cells was measured with an EZ-Detect™Ras Activation kit (PIERCE, Rockford, IL), following the manufacturer's instructions. Briefly, the cells were lyzed by adding 1 ml Lysis/Binding/Wash Buffer. The lysates were briefly sonicated and centrifuged. To 500 μl lysate were added 10 μl 0.5 M EDTA (pH 8.0) and 5 μl 10 mM GTPγS. Incubation followed at 30°C for 15 min. Reaction was stopped on ice by adding 32 μl 1 M $MgCl_2$. The solution was incubated with GST-Raf1-RBD (Ras binding domain, residues 1–149) in the presence of a SwellGel® Immobilized Glutathione Disc. The pulled-down active Ras was detected by Western blot analysis using anti-Ras Antibody in the kit.

Cholesterol measurement

Confluent cultures of PC-12 cells in 75 cm^2 culture flasks were incubated for 24 h in DMEM containing 1% FBS with 1 μM simvastatin. After washing with DMEM, the cells were scraped and centrifuged. The pellet was suspended and extracted 3 times with 100 μl a lysis buffer (PBS/1.25 mM sodium cholate/0.025% Triton X-100). The cholesterol content was assayed in 96-well plates with a Cholesterol E-test Wako (Wako Pure Chemical Co., Osaka, Japan), following the manufacturer's instructions.

Statistical analysis

All values are expressed as the mean ± S.D., and significant levels between group were assessed by ANOVA and Bonferroni's test. A 5% level of probability was considered to be significant.

Results

The time-dependent effects of statins on the survival and morphology of undifferentiated PC-12 Cells

Fig. 1 shows the effects of statins on the survival of undifferentiated PC-12 cells cultured in DMEM containing 1% FBS. Incubating the cells for ~48 h with 0.1-1 μM each statin had little or no effect on their survival. Higher concentrations (≥10 μM) of mevastatin and lovastatin induced cell death in varying degrees. We next examined the effects of these statins on the cell morphology of undifferentiated PC-12 cells. As shown in Fig. 2, after incubation for 24 h with 1μM lovastatin and simvastatin, the drugs exhibited neurite-forming activity in PC-12 cells. Mevastatin had no effect. The neurites formed by simvastatin treatment remained intact at 72 h, while in the lovastatin-treated cells, neurite loss was considerable and 35% of the cells died. These data suggest that, of the three statins, simvastatin at 1 μM exerts the most effective neurite-forming

activity without killing the cells. Hereafter, special emphasis is placed on the NGF-like activity of simvastatin.

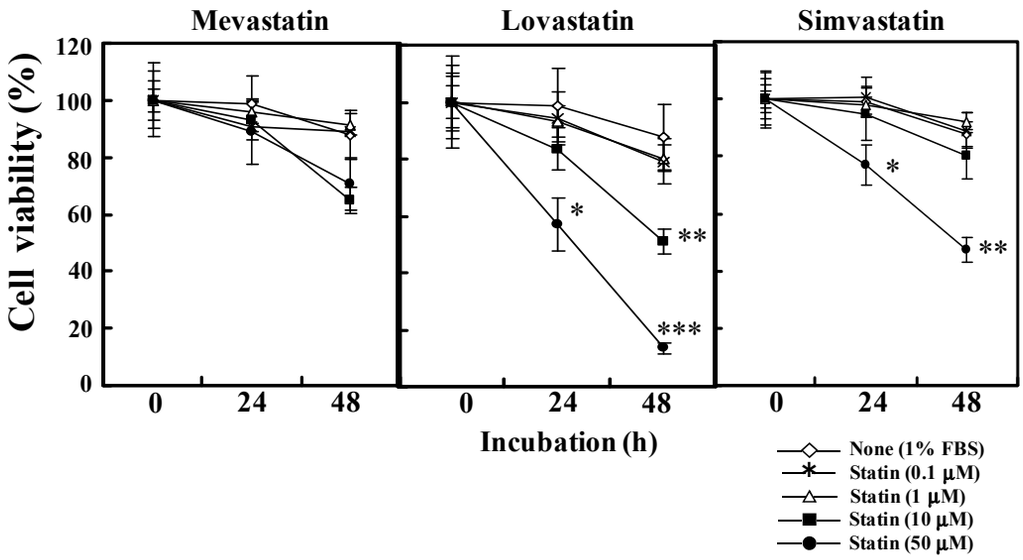

Figure 1. The Time-dependent effects of statins on the survival of undifferentiated PC-12 cells. The cells in 96-well plates were exposed for 24 or 48 h at 37°C to 0.1 -50 μM statin in DMEM containing 1% FBS. Cell viability was measured by using a Cell Counting Kit-8. Each bar represents the mean ± S.D. (n= 3). **P*< 0.05; ***P*< 0.01; ****P*< 0.001 *vs* no statin.

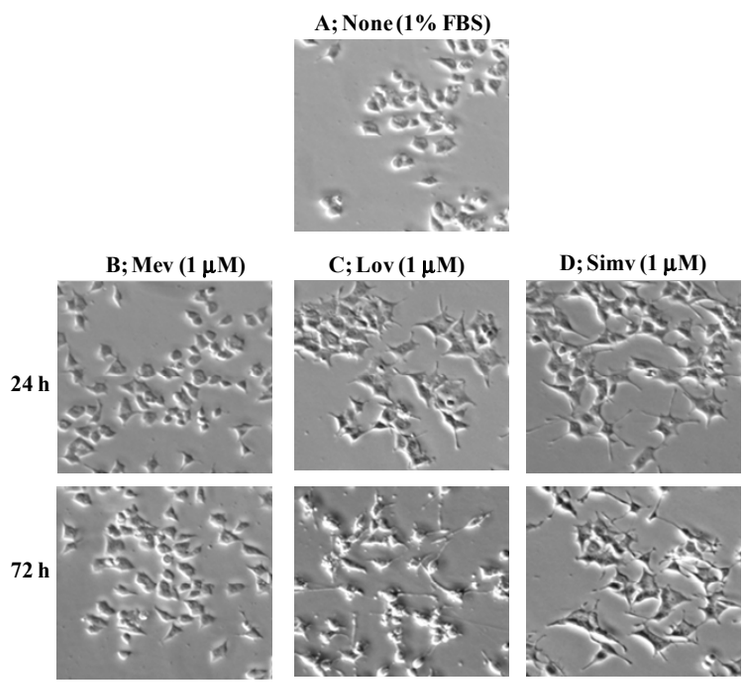

Figure 2. The effects of statins on the morphology of undifferentiated PC-12 cells. The cells in 6-well plates were exposed for 24 or 72 h to 1 μM statin in DMEM containing 1% FBS. Morphological changes were assessed by phase-contrast microscopy.

Simvastatin's effect on NGF-induced neurite outgrowth and survival signaling

Fig. 3A shows the morphologies of PC-12 cells treated for 72 h with NGF alone and with NGF plus 1 μM simvastatin. Simvastatin promoted the NGF-induced neurite outgrowth more potently than an additive effect. As shown in Fig. 3B, the total length of neuronal networks formed by simvastatin treatment was almost the same as that formed by NGF treatment. NGF plus simvastatin caused a 2.5-fold increase in neurite formation, compared to NGF alone. We next examined the effects of NGF, simvastatin and their combination on the phosphorylated state of ERKs, survival signals in PC-12 cells.

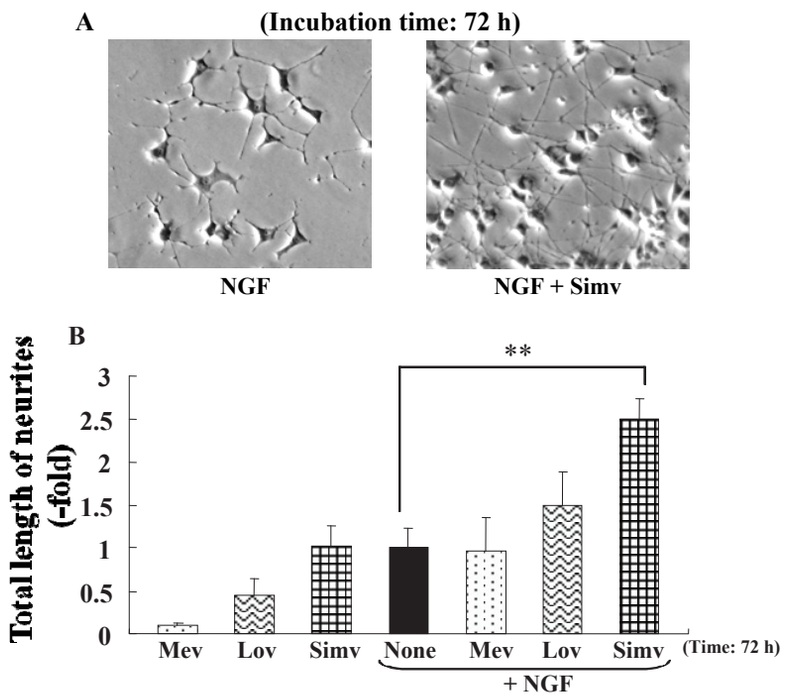

Figure 3. (A) Morphologies of PC-12 cells treated for 72 h with NGF alone and NGF plus 1 μM simvastatin, respectively. (B) Total length of the neurites formed by PC-12 cells treated for 72 h with statin alone, NGF alone, or NGF plus statin. The length of neuronal networks was quantified using an NIH Image. Each bar represents the mean ± S.D. of (n= 3). ***P*< 0.01, compared to NGF alone.

As shown in Fig. 4A, the total levels of phosphorylated ERKs in the cells peaked at 6 h after exposure to NGF, simvastatin, or both in combination, and then gradually decreased. The addition of 1 μM simvastatin clearly increased the NGF-induced levels of phosphorylated ERKs and sustained them for a considerable period of time. In contrast, 1 μM simvastatin alone did not affect the phosphorylated levels of Akt, which is also survival signal in the cells (Fig. 4B). These results suggest that simvastatin at limited low concentrations can promote neurite outgrowth and survival of PC-12 cells at

least via activation of the MAPK/ERK pathway.

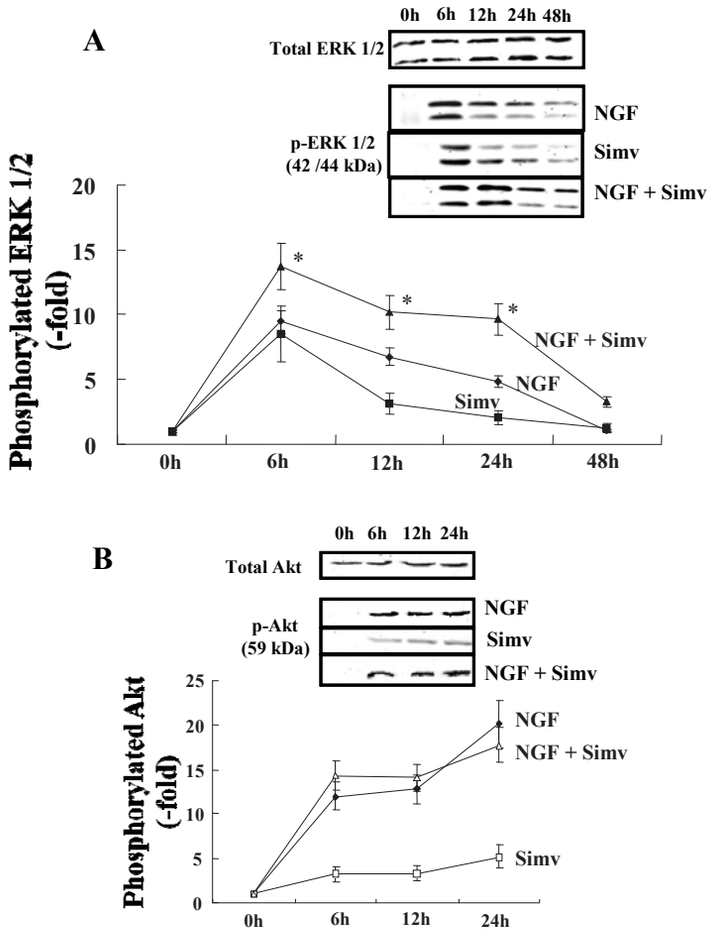

Figure 4. The time-dependent effects of NGF, simvastatin and their combination on the phosphorylations of ERKs (A) and Akt (B) in PC-12 cells. The cells were incubated at 37℃ for ~48 h with NGF alone, 1 μM simvastatin alone, or NGF plus 1 μM simvastatin. The phosphorylated ERKs 1/2 and Akt were made visible by Western blotting and the bands were quantified using an NIH image. Each bar represents the mean ± S.D. (n= 3). **P*< 0.05, compared to NGF alone.

Fig. 5 shows the effects of PD98059 and LY294002, which are MEK and PI-3K inhibitors, respectively, on the NGF/simvastatin-induced neurite outgrowth of PC-12 cells. The panels A–D show the morphologies of PC-12 cells treated for 24 h with NGF and simvastatin or plus the inhibitors. As shown in Fig. 5E, the total length of neuronal networks formed by the treatment with simvastatin and NGF was almost the same as that formed by the treatment with simvastatin, NGF and LY294002. On the other hand, simvastatin, NGF and PD98059 caused a 2-fold decrease in neurite formation, compared to simvastatin plus NGF. These data suggest that simvastatin has the ability to positively modulate the MAPK/ERK pathway, which plays a key role in neurite

outgrowth, rather than the PI-3K/Akt pathway in PC-12 cells. However, at least both pathways are required in PC-12 cells for NGF-mediated survival, because inhibition of the two pathways by PD98059 and LY294002 induced cell death (Fig. 5D).

Simvastatin's effect on cholesterol synthesis and Ras activation in PC-12 Cells

As shown in Fig. 6A, treatment with 1 μM simvastatin and NGF for 24 h did not alter the cholesterol content in the cells. Fig. 6B shows the time course of Ras activation in PC-12 cells treated with NGF alone or NGF plus simvastatin. This suggests that the NGF-induced Ras activation was also unaffected by the treatment with 1 μM simvastatin. However, it remains unknown whether the unchanged cholesterol content in the cells was resulted from the cell starvation or from the statin's modulation of other biochemical target(s).

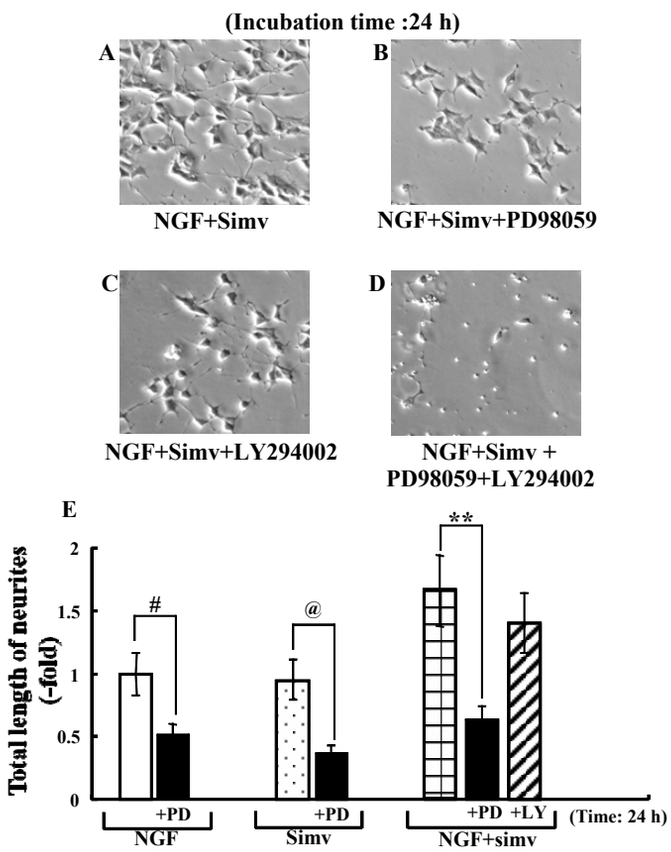

Figure 5. Morphologies of PC-12 cells treated for 24 h with NGF and 1 μM simvastatin or with the two agents plus 100 μM inhibitor(s). Panel A, NGF + simvastatin; B, NGF + simvastatin + PD98059; C, NGF + simvastatin + LY294002; D, NGF + simvastatin + LY294002 + PD98059; E, Total length of the neurites formed by these cells. The length of neuronal networks was quantified using an NIH Image. Each bar represents the mean ± S.D. (n= 3). $^{\#}P< 0.05$, compared to NGF alone, $^{@}P< 0.05$, compared to simvastatin alone, $^{**}P< 0.01$, compared to NGF plus simvastatin.

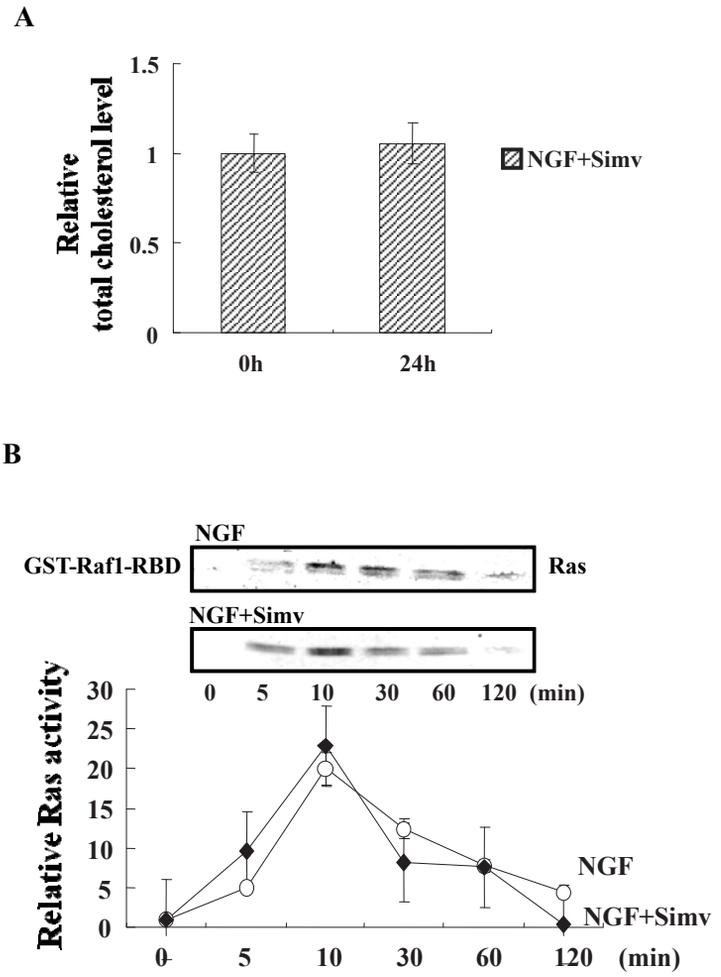

Figure 6. (A) Cholesterol content in PC-12 cells treated with NGF plus simvastatin for 24 h. (B) The time course of Ras activation in PC-12 cells treated with NGF alone or NGF plus simvastatin. Each bar represents the mean ± S.D. (n= 3).

Discussion

While the specific targets of statins or their second messengers within the brain are not yet known, the drugs have been reported to reduce the incidence of Alzheimer's disease (Jick, Zornberg *et al.,* 2000; Wolozin, Kellman *et al.,* 2000**)** and to affect cholesterol homeostasis in the central nervous system (Fassbender, Stroick *et al.,* 2002). In animal models, reducing the cellular cholesterol levels of hippocampal neurons with lovastatin inhibits the formation of β-amyloid, suggesting that cholesterol is required for the abnormal protein formation (Simons, Keller *et al.,* 1998). Schulz, Bösel *et al.* (2004) have recently demonstrated that using atorvastatin to inhibit mevalonate synthesis in PC-12 cells treated with NGF or in primary cortical neurons causes neurite loss and cell

death, because geranylgeranylpyrophosphate, which may be critical for neurite outgrowth and maintenance, is consumed to compensate for the lack of mevalonate. It is true that inhibition of HMG-CoA reductase affects survival of neurons by altering the cholesterol metabolism (Fan, Yu *et al.,* 2002; Meske, Albert *et al.,* 2003; Michikawa and Yanagisawa, 1999). However, statins have pharmacological properties beyond that of simply reducing cholesterol levels. Simvastatin induces neurite outgrowth (Sato-Suzuki and Murota, 1996) and also nitric oxide production in PC-12 cells (Sano, Sato-Suzuki *et al.,* 1998), but it is unknown whether these effects are a result of changes in the cell cholesterol homeostasis.

The statins used in this study, simvastatin, lovastatin and mevastatin, are highly lipophilic and pass the blood-brain barrier easily (Saheki, Terasaki *et al.,* 1994). We found that high concentrations of these statins (≥10 μM) reduced cell survival rates, although their cytotoxic potencies differed. Their cytotoxic effects may be partly explained as follows. Because undifferentiated PC-12 cells are mitotic, they require nonsterol isoprenoid products for the DNA synthesis. The inhibition of HMG-CoA reductase causes a deficiency of the intermediate metabolites in cholesterol synthesis and induces cell death (Padayatty, Marcelli *et al.,* 1997; Sato, Isobe *et al.,* 1996). However, low concentrations (≤1 μM) of these statins had no effect on the cell' survival. Statins decrease isoprenoids products of mevalonate with a decrease in cholesterol synthesis. Farnesylpyrophosphate, one of the isoprenoids, provides a lipophilic anchor which is essential for membrane attachment and the biological activity of small GTP-binding proteins including Ras (Meske, Albert *et al.,* 2003). To play a role in cell signaling, Ras requires farnesylpyrophosphate and must be translocated from the cytoplasm to the cell membrane (Meske, Albert *et al.,* 2003). If simvastatin reduces the intracellular levels of cholesterol in PC-12 cells, mevalonate-derived isoprenoids may also be decreased. The decrease in farnesylpyrophosphate leads to a reduced translocation and activation of Ras. Our results demonstrated that the treatment of the cells with 1 μM simvastatin had no effect on the cholesterol content and Ras activation. Atorvastatin inhibits the activity of HMG-CoA reductase almost as effectively as mevastatin; yet in low concentrations, it barely affected the cholesterol metabolism in primary cortical neurons (Schulz, Bösel *et al.,* 2004). Therefore, our results suggest that

in PC-12 cells, statins in concencrations of ≤1 μM, might not alter cholesterol metabolism so as to induce cell death.

We have demonstrated that at a concentration of 1 μM, simvastatin induces neurite outgrowth in PC-12 cells as does NGF and that the neurites formed are preserved at 72 h. However, lovastatin exerted both neurite-forming activity and cytotoxicity, having killed 35% of the cells at the end of 72 h. Mevastatin showed neither neurite-forming activity nor cytotoxicity in the same circumstances. The three statins have a common lactone structure. The lactone ring, which lactacystin also possesses, is reported to contribute to neurite-forming activity (Nagamitsu, Sunazuka *et al.,* 1995). However, mevastatin did not induce the formation of neurites, although it possesses the same lactone structure. A key difference in the structures of the three statins is the number of methyl moieties. Simvastatin, lovastatin and mevastatin have two, one and zero methyl groups, respectively. Simvastatin, which has two methyl groups, showed the most potent neurite-forming activity, while mevastatin, which has no methyl group, showed none. However, it is unknown how the methyl group contributes to the formation of neurites.

We have demonstrated that in PC-12 cells, simvastatin and NGF can jointly promote the phosphorylation (activation) of ERKs, but not of Akt. The MAPK/ERK pathway is important in mediating NGF-induced neurite outgrowth response in PC-12 cells (Creedon, Johnson *et al.,* 1996; Klesse, Meyers *et al.,* 1999). Simvastatin in combination with NGF increased levels of phosphorylated ERKs and extended their duration (Kumano, Mutoh *et al.,* 2000). NGF, acting through the TrkA receptor, induces a sustained activation of ERKs that is required for differentiation. The enhancement of ERK signaling resulted in the promotion of neurite outgrowth. The binding of NGF to cell surface receptor TrkA leads to activation of MEK1 and ERK1/2. The activated ERKs move to the nucleus and mediate transcription factors required for the neuron's survival and gene expression (Riccio, Ahn *et al.,* 1999). Neurite outgrowth in PC-12 cells is also induced by an NGF-independent elevation of intracellular cAMP levels and followed by activation of MEK and ERK (Frodin, Peraldi *et al.,* 1994). It remains unknown how simvastatin activated the ERKs in PC-12 cells: however, the cAMP-dependent mechanism may be excluded, because the drug had no effect on

intracellular cAMP levels in endothelial cells (Van Nieuw, Vermeer *et al.,* 2000).

In conclusion, we have demonstrated that simvastatin has the ability to aid NGF-related neuritogenesis and maintenance at limited low concentrations (~1 μM). Many investigations of statin's effects on neuronal cells were also carried out at a concentration range of 0.1–50 μM (Sato-Suzuki and Murota, 1996; Schulz, Bösel *et al.,* 2004; Sano, Sato-Suzuki *et al.,* 1998). However, according to the interview form of simvastatin (Mr= 418.57), the peak concentration in plasma of this drug in patients who received 20 mg/day is ~4 ng/ml (0.01 μM). There is no relevancy between the neurite outgrowth activity of simvastatin and the dosage used at the pharmaceutical levels. However, further studies on the NGF-like activity of simvastatin, which is independent of its major effect on cholesterol synthesis, may provide a new therapeutic approach to control age-related neuronal cell death.

Acknowledgements

This work was supported in part by funds from Central Research Institute of Fukuoka University and from MEXT. HAITEKU (2002-2006). Our thanks to Mr. Steven Sabotta for reading the manuscript.

References

Creedon, DJ., Johnson, EM. and Lawrence, JC. (1996). Mitogen-activated protein kinase-independent pathways mediate the effects of nerve growth factor and cAMP on neuronal survival. J. Biol. Chem. 271: 20713–20718.

Fan, QW., Yu, W., Gong, JS., Zou, K., Sawamura, N., Senda, T., Yanagisawa, K. and Michikawa, M. (2002). Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J. Neurochem. 80: 178–190.

Fassbender, K., Stroick, M., Bertsch, T., Ragoschke, A., Kuehl, S., Walter, S., Walter, J., Brechtel, K., Muehlhauser, F., von Bergmann, K. and Lütjohann, D. (2002). Effects of statins on human cerebral cholesterol metabolism and secretion of Alzheimer amyloid peptide. Neurology. 59: 1257–1258.

Föcking, M., Besselmann, M. and Trapp, T. (2004). Statins potentiate caspase-3 activity in immortalized murine neurons. Neurosci. Lett. 355: 41–44.

Frodin, M., Peraldi, P. and Obberghen, EV. (1994). Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. J. Biol. Chem. 269: 6207–6214.

Gaist, D., Jeppesen, U., Andersen, M., Garcia, LA., Hallas, RJ. and Sindrup, SH. (2002). Statins and risk of polyneuropathy: a case-control study (comment). Neurology. 58: 1333–1337.

Jeppesen, U., Gaist, D., Smith, T. and Sindrup, SH. (1999). Statins and peripheral

neuropathy. Eur. J. Clin. Pharmacol. 54: 835–838.
Jick, H., Zornberg, GL., Jick, SS., Seshadri, S. and Drachman, DA. (2000). Statins and the risk of dementia. Lancet. 356: 1627–1631.
Klesse, LJ., Meyers, KA., Marshall, CJ. and Parade, LF. (1999). Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. Oncogene. 18: 2055–2068.
Kumano, T., Mutoh, T., Nakagawa, H. and Kuriyama, M. (2000). HMG-CoA reductase inhibitor induced a transient activation of high affinity nerve growth factor receptor, Trk, and morphological differentiation with fatal outcome in PC12 cells. Brain Res. 859: 169–172.
Meske, V., Albert, F., Richter, D., Schwarze, J. and Ohm, TG. (2003). Blockade of HMG-CoA reductase activity causes changes in microtubule-stabilizing protein tau via suppression of geranylgeranylpyrophosphate formation: implications for Alzheimer's disease. Eur. J. Neurosci. 17: 93–102.
Michikawa, M. and Yanagisawa, K. (1999). Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. J. Neurochem. 72: 2278–2285.
Nagamitsu, T., Sunazuka, T., Stump, H., Obata, R., Arima, S., Matsuzaki, K., Tanaka, H. and Omura, S. (1995). Structure-activity relationships of lactacystin, the first non-protein neurotrophic factor. J. Antibiotics. 48: 747–748.
Padayatty, S., Marcelli, T., Shao, TC. and Cunningham, GR. (1997). Lovastatin-induced apoptosis in prostate stromal cells. J. Clin. Endocrinol. Metab. 82: 2358–2361.
Riccio, A., Ahn, S., Davenport, CM., Blendy, JA. and Ginty, DD. (1999). Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science. 286: 1434–1439.
Saheki, A., Terasaki, T., Tamai, I. and Tsuji, A. (1994). In vivo and in vitro blood-brain barrier transport of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. Pharm. Res. 11: 305–311.
Sano, M., Sato-Suzuki, I., Fujita, H., Morita, I., Nagano, M. and Murota, S. (1998). NO is not involved in the simvastatin induced cell division and differentiation in PC 12 cells. Neurosci .Lett. 243: 73–76.
Sato, T., Isobe, H., Ayukawa, K., Sakai, H. and Nawara, H. (1996). The effects of pravastatin, an HMG-CoA reductase inhibitor, on cell viability and DNA production of rat hepatocytes. Life Sci. 59: 1103–1108.
Sato-Suzuki, I. and Murota, S. (1996). Simvastatin inhibits the division and induces neurite-like outgrowth in PC12 cells. Neurosci. Lett. 220: 21–24.
Schulz, JG., Bösel, J., Stoeckel, M., Megow, D., Dirnagl, U. and Endres, M. (2004). HMG-CoA reductase inhibition causes neurite loss by interfering with geranylgeranylpyrophosphate synthesis. J. Neurochem. 89: 24–32.
Simons, M., Keller, P., De Strooper, B., Beyreuther, K. and Dotti, CG. (1998). Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons. Proc. Natl. Acad. Sci. USA. 95: 6460–6464.
Sinzinger, H., Mayr, F., Schmid, P., Granegger, S., O'Grady, J. and Peskar, BA. (1994). Sleep disturbance and appetite loss after lovastatin. Lancet. 343: 973.
Van Nieuw, Amerongen, GP., Vermeer, MA., Negre-Aminou, P., Lankelma, J., Emeis, JJ. and van Hinsbergh, VW. (2000). Simvastatin improves disturbed endothelial

barrier function. Circulation. 102: 2803–2809.
Veillard, NR., and Mach, F. (2002). Statins: The new aspirin? Mol. Life Sci. 59: 1771–1786.
Wolozin, B., Kellman, W., Ruosseau, P., Celesia, GG. and Siegel, G. (2000). Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Arch. Neurol. 57: 1439–1443.