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Simvastatin enhances hippocampal long-term potentiation in C57BL/6 mice

Robert A. Mans^{1,2}, Nazma Chowdhury², Dongfeng Cao², Lori L. McMahon^{1,3}, and Ling Li^{1,2}

¹Department of Neurobiology, University of Alabama at Birmingham, Birmingham, Alabama

²Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

³Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama

Abstract

Statins inhibit 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA), the rate-limiting enzyme in the cholesterol biosynthetic pathway, and they are widely used to control plasma cholesterol levels and prevent cardiovascular disease. However, emerging evidence indicates that the beneficial effects of statins extend to the central nervous system. Statins have been shown to improve the outcome of stroke and traumatic brain injury, and statin use has been associated with a reduced prevalence of Alzheimer's disease (AD) and dementia. However, prospective studies with statins in AD have produced mixed results. Recently, we reported that simvastatin, a widely used statin in humans, enhances learning and memory in non-transgenic mice as well as in transgenic mice with AD-like pathology on a mixed genetic background. However, the cellular and molecular mechanisms underlying the beneficial effects of simvastatin on learning and memory remain elusive. The present study was undertaken to investigate the effect of acute simvastatin treatment on hippocampal long-term potentiation (LTP), a cellular model of learning and memory, in brain slices from C57BL/6 mice. Our results demonstrate that a prolonged *in vitro* simvastatin treatment for 2–4 hrs, but not a short-term 20-min exposure, significantly increases the magnitude of LTP at CA3-CA1 synapses without altering basal synaptic transmission or the paired-pulse facilitation ratio in hippocampal slices. Furthermore, we show that phosphorylation of Akt (protein kinase B) is increased significantly in the CA1 region following 2-hour treatment with simvastatin, and that inhibition of Akt phosphorylation suppresses the simvastatin-induced enhancement of LTP. These findings suggest activation of Akt as a molecular pathway for augmented hippocampal LTP by simvastatin treatment, and implicate enhancement of hippocampal LTP as a potential cellular mechanism underlying the beneficial effects of simvastatin on cognitive function.

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Corresponding author: Ling Li, DVM, PhD, Department of Medicine, University of Alabama at Birmingham, 1808 7th Ave S, BDB 658, Birmingham, AL 35294-0012, Phone 205-934-1889, Fax 205-975-8079, lili@uab.edu.

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Keywords

statins; synaptic plasticity; learning and memory; cognitive function; protein phosphorylation; Alzheimer's disease

Statins inhibit the rate-limiting reaction in the mevalonate biosynthetic pathway for cholesterol, which entails conversion of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) to mevalonate via HMG-CoA reductase (Brown and Goldstein, 1986). Inhibiting this reaction not only reduces *de novo* cholesterol synthesis, but also prevents the formation of isoprenoid intermediates, such as farnesyl-pyrophosphate (PP) and geranylgeranyl-PP, which serve as lipid attachments for various intracellular signaling molecules like the small GTP-binding proteins Rho, Ras and Rac (Liao, 2002, Vaughan, 2003). The subcellular localization, intracellular trafficking, and function of these GTPases depend on their isoprenylation state. Consequently, inhibition of isoprenoid production by statins induces pleiotropic effects that go beyond the reduction of cholesterol. A growing body of literature supports the notion that these pleiotropic effects extend to brain.

A diverse characterization of the cognitive effects of statins has been published, with many studies focusing on the potential therapeutic benefits of statins for the treatment of stroke or traumatic brain injury (Balduino et al., 2003, Chen et al., 2003, Lu et al., 2007a, Wu et al., 2008). In rat models, chronic administration of atorvastatin or simvastatin following traumatic brain injury have been shown to improve rehabilitation of spatial memory (Lu et al., 2007a), reduce inflammatory cytokine production (Balduino et al., 2003), and improve cerebral blood flow to the injury site (Chen et al., 2003). Furthermore, neurogenesis in the dentate gyrus (Chen et al., 2003, Lu et al., 2007a), angiogenesis and neurite outgrowth (Pooler et al., 2006) are stimulated by chronic ($\geq$ seven days) statin treatment.

Statins have received much attention in the field of Alzheimer's disease (AD), as well, due in large part to findings from epidemiological studies indicating a lower prevalence of AD/dementia in statin-prescribed populations (Jick et al., 2000, Wolozin et al., 2000), suggesting a neuroprotective role for statins. However, prospective studies with statins have produced mixed results. While some studies showed no protection of statins in preventing AD in a group of patients at risk for cardiovascular disease (Collins et al., 2002, Shepherd et al., 2002), others reported that statin (simvastatin or atorvastatin) treatment improved cognitive function in normocholesterolemic patients (Simons et al., 2002) or slowed the decline in cognitive function of AD patients (Sparks et al., 2005). Also, some studies showed a decrease of amyloid- β peptide (A β) after statin treatments (Buxbaum et al., 2002, Sjogren et al., 2003), whereas others found no effects of statin treatments on A β levels (Fassbender et al., 2002, Hoglund et al., 2004, Hoglund et al., 2005). Subsequent work has examined the contribution of the anti-inflammatory properties of statins. Namely, pro-inflammatory responses of microglia after A β exposure are ameliorated after statin treatment *in vitro* (Cordle and Landreth, 2005) and *in vivo* (Clarke et al., 2008). Also, statins protect cultured cortical neurons from excitotoxicity after exposure to *N*-methyl *D*-aspartate (NMDA) (Zacco et al., 2003) and monosodium glutamate (Bosel et al., 2005). In addition, atorvastatin increases long-term potentiation (LTP) in the hippocampus of rats following acute *in vivo* administration of amyloid- β peptide (Clarke et al., 2007). However, these potentially beneficial effects of statins are countered by a report that acute *in vitro* treatment of mouse hippocampal slices with mevastatin (also known as compactin; a statin not approved for human use) inhibits LTP at CA3-CA1 synapses (Kotti et al., 2006). While statins share a common mechanism for inhibiting cholesterol synthesis, differences exist regarding their potency (rosuvastatin > atorvastatin > simvastatin > lovastatin = pravastatin > fluvastatin) and their ability to cross the blood-brain barrier (simvastatin > atorvastatin and rosuvastatin) (Jones, 2003). Recently, we reported that chronic simvastatin treatment *in vivo* enhances

hippocampal-dependent learning and memory in non-transgenic mice as well as in a transgenic mouse model of AD (Li et al., 2006).

The goal of the present study was to ascertain whether the pleiotropic effects of statins extend to the cellular mechanisms of synaptic plasticity that contribute to hippocampal-dependent learning. To this end, we sought to determine whether acute *in vitro* exposure of hippocampal slices to simvastatin increases the magnitude of NMDA receptor-dependant LTP at CA3-CA1 synapses in slices from C57BL/6 mice. Our results demonstrate that a prolonged *in vitro* simvastatin treatment for 2-4 hr, but not a short-term 20-min exposure, increases the magnitude of LTP at CA3-CA1 synapses without changing basal synaptic transmission or the paired-pulse facilitation (PPF) ratio in hippocampal slices. Furthermore, we show that simvastatin treatment for 2 hr, but not 20 min, significantly increases the phosphorylation of protein kinase B (Akt). These findings suggest that modulation of hippocampal LTP and related signaling pathways could be one of the underlying cellular and molecular mechanisms by which simvastatin treatment enhances learning and memory.

EXPERIMENTAL PROCEDURES

Animals

Three- to four-month-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME; stock no. 000664) were used in this study. The mice were housed in a specific-pathogen-free facility under veterinary supervision at an ambient temperature of 22-23 °C and under a 12:12-hr light/dark cycle. The mice were allowed *ad libitum* access to food and water. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Preparation of simvastatin solution for electrophysiology experiments

Simvastatin was kindly provided by Merck & Co., Inc., West Point, PA. Prior to its use in experiments, simvastatin was converted from its inactive lactone prodrug form to its active dihydroxy open acid form by alkaline hydrolysis (first dissolving 50 mg of the compound in 1 ml of ethanol (100%) and then adding 0.813 ml of 1 N NaOH). This stock solution was stored in aliquots at -20°C (for up to 1 month). On the day of use, the simvastatin stock solution was neutralized with 1 N HCl to pH of 7.4 and diluted in artificial cerebral spinal fluid (aCSF). The final concentration of simvastatin used in the aCSF was 10 µM. In addition, a vehicle solution without simvastatin was prepared and added to the aCSF to serve as controls.

Slice preparation and electrophysiology

Hippocampal slices (400 µM) were prepared from male C57BL/6 mice using methods described previously (Smith and McMahon, 2006) with modifications. Briefly, mice were anesthetized with isoflurane and decapitated. Their brains were removed and immersed in ice-cold "high-sucrose" aCSF composed of (in mM): NaCl 85; KCl 2.5; MgSO₄ 4; CaCl₂ 0.5; NaH₂PO₄ 1.25; NaHCO₃ 25; glucose 25; sucrose 75; 290-300 mOsm. This sucrose-based aCSF, which contains less Na⁺ and Ca²⁺ and higher Mg²⁺ than the regular sodium-based aCSF promotes neuronal survival during the slicing procedure by reducing excitotoxicity (Kuenzi et al., 2000). Coronal slices of the dorsal hippocampus were cut on a vibratome (Vibratome, St. Louis, MO) and incubated in sucrose-based aCSF for ten min, then for ≥ one hr in regular aCSF containing (in mM): NaCl 119; KCl 2.5; CaCl₂ 2.5; MgSO₄ 1.3; NaH₂PO₄ 1; NaHCO₃ 26; and glucose 10 saturated with 95% O₂-5% CO₂ (pH 7.4). To record field excitatory postsynaptic potentials (fEPSPs), slices were placed in a submersion recording chamber and continuously perfused at 3-5 ml/min with aCSF warmed to 26-28°C. CA1 extracellular dendritic fEPSPs were recorded (Axopatch 200B, Molecular Devices, Sunnyvale, CA) using standard methods (Smith and McMahon, 2005). Stimulus frequency was 0.1 Hz (100-µs

duration), and stimulus intensity was adjusted to yield fEPSPs with amplitudes of 0.5–0.8 mV. Schaffer collaterals were stimulated with a bipolar tungsten stimulating electrode placed in CA1 stratum (s.) radiatum, and fEPSPs were recorded using a glass microelectrode filled with aCSF, also placed in CA1 s. radiatum. If stable fEPSPs were maintained for at least 20 minutes, NMDA receptor-dependent LTP was induced with a high-frequency stimulation (HFS) protocol (four 0.5-s 100 Hz trains applied at a 20-s interval) (Smith and McMahon, 2005). The stimulus intensity was increased to 1.5 times the baseline intensity during the HFS to ensure strong postsynaptic depolarization and NMDA receptor activation, and was returned to the baseline intensity immediately after HFS.

Simvastatin treatment

Two simvastatin treatment protocols were used: 1) a 20-minute bath application of simvastatin during collection of baseline transmission and 2) a 2–4 hr simvastatin incubation prior to HFS induction of LTP; simvastatin was bath applied throughout the recording period.

Twenty-minute treatment—First, stable baseline transmission was recorded for at least 10 min in regular aCSF, after which time bath application of either simvastatin (10 μ M) or vehicle commenced and continued for 20 min. Once a stable 20 min baseline in simvastatin was acquired, HFS was delivered to induce LTP. Simvastatin (or vehicle) perfusion continued throughout the experiment.

Two- to four-hour incubation—Following slicing and 10-min incubation period in sucrose-based aCSF, half of the slices were incubated at room temperature in regular aCSF supplemented with simvastatin (10 μ M), and half were incubated in regular aCSF supplemented with vehicle. The experimenter was blinded to the identity of the solutions. The slices were incubated in these solutions for a period $\geq$ one hour. Following this, recordings were performed, alternating between simvastatin- and vehicle-treated slices in an interleaved fashion. Importantly, the order of recordings was reversed between mice, to ensure that the results are not biased towards longer incubation times in simvastatin or vehicle. Because experiments were interleaved between the two conditions, and the order of the experiments was reversed between animals, the simvastatin incubation times (time from onset of drug exposure to HFS) varied between 2 to 4 hours. Simvastatin (or vehicle) was perfused continuously throughout the recording period. For experiments with a phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Sigma, St. Louis, MO), LY294002 stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and added to the aCSF in a final concentration of 20 μ M containing 0.1% DMSO. In these experiments, the same concentration of DMSO (0.1%) was added to vehicle as a control.

In a separate set of experiments, slices were prepared in regular aCSF, rather than in the sucrose-based aCSF. In these experiments, slices also underwent the 2–4 hour simvastatin incubation period as described above.

Immunoblot analysis

Hippocampal slices were prepared as described above for LTP experiments. For each mouse, 6 hippocampal slices were obtained: 3 slices were incubated at room temperature in regular aCSF supplemented with simvastatin (10 μ M), and 3 slices were incubated in regular aCSF supplemented with vehicle. After 20 min or 2 hr, the CA1 region was dissected and pooled from 3 slices followed by an immediate homogenization in SDS sample buffer (Invitrogen, Carlsbad, CA) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). For some slices, the entire hippocampal area was dissected out and homogenized in SDS sample buffer. After a brief sonication, protein concentrations were determined by the Bio-Rad Protein Assay

(Bio-Rad Laboratories, Hercules, CA). Twenty-five μg of proteins for each sample were separated by SDS-PAGE, and blotted to PVDF membranes. The membranes were incubated with primary antibodies followed by biotinylated or HRP-conjugated secondary antibodies. Signal was detected by the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and quantified by densitometric scanning using the LabWorks image acquisition and analysis software (UVP Inc., Upland, CA). For a loading control, the blots were stripped and re-probed with a mouse anti-tubulin monoclonal antibody (Sigma, St. Louis, MO). Primary antibodies used for immunoblot analysis are rabbit polyclonal antibodies against Akt (protein kinase B) (Santa Cruz) and phospho-Akt (p-Akt, Ser473) (Cell Signaling, Danvers, MA).

Data analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Comparison of data from different treatment groups was performed by two-tailed Student's *t* test (for normally distributed data) or Mann-Whitney rank sum test (for non-normally distributed data). $P < 0.05$ was considered statistically significant. Data from electrophysiology experiments were filtered at 3 kHz, digitized at 10 kHz, and acquired using LabVIEW data acquisition software (Richard Mooney, Duke University). The slope of the rising phase of the fEPSP was measured and plotted versus time. Each point represents the average of five raw data points. To determine the magnitude of LTP, the slopes of the rising phase of the fEPSPs were normalized to baseline, and the fEPSPs between thirty-five and forty minutes post-HFS were averaged. For the 20-min treatment and incubation experiments with slices prepared in sucrose-based aCSF, data were analyzed in pairs such that experiments were not included in the analyzed data set unless a pair of simvastatin- and vehicle-treated experiments were successfully completed from the same mouse brain. This requirement controlled for inter-animal variability as well as potential differences in slice health between batches of slices. For incubation experiments from slices prepared in regular aCSF, pairs of data were not always achieved, so LTP values from all animals were included in the final data set.

RESULTS

Twenty-minute simvastatin treatment is not sufficient to affect the magnitude of hippocampal LTP

We tested the effects of acute simvastatin exposure *in vitro* on synaptic plasticity in mouse hippocampus. LTP experiments were performed at CA3-CA1 synapses in coronal slices from C57BL/6 mice. Upon completion of the resting period ($\geq$ one hr), slices were moved to the recording chamber, and a stable (>20 min) baseline was obtained in regular aCSF. Simvastatin or vehicle was then bath applied and synaptic transmission was acquired for an additional 20 min. There was no effect of simvastatin on basal synaptic transmission, as neither the paired-pulse facilitation (PPF) ratio (Fig. 1A) nor the fEPSP slope (Fig. 1B) changed significantly during simvastatin perfusion. Following acquisition of a stable baseline, high frequency stimulation (HFS; 4 trains, 100 Hz, 0.5 s in duration, 20 s interval) was applied to induce LTP. As shown in Fig 1B, there was no significant effect of the 20-min simvastatin treatment on the magnitude of LTP expressed (Fig. 1B) ($147 \pm 11\%$ and $137 \pm 12\%$ of baseline fEPSP slope in simvastatin-treated slices ($n = 7$ slices/6 mice) and in vehicle-treated slices ($n = 7$ slices/6 mice), respectively; $P > 0.05$).

Two- to four-hour simvastatin treatment significantly increases the magnitude of hippocampal LTP

To determine whether a longer simvastatin exposure would affect the magnitude of LTP, hippocampal slices from C57BL/6 mice were incubated in regular aCSF supplemented with simvastatin or vehicle. Incubation began immediately after the 10-min post-slicing resting

period in sucrose-based aCSF and continued until slices were transferred to the recording chamber. The recording chamber was continuously perfused with simvastatin- or vehicle-containing aCSF. Because experiments from simvastatin-treated slices were interleaved with experiments from vehicle-treated slices from the same animal using the same electrophysiology recording setup, the simvastatin incubation times varied from 2-4 hours before inducing LTP with HFS. Consistent with the results from the 20-min treatment experiments, basal transmission was unaffected by longer incubation in simvastatin, as there was no difference in the input/output relationship (Fig. 2A). Furthermore, there was no difference in the PPF ratio (Fig. 2B). However, in contrast to the 20-min treatment, 2-4-hr simvastatin incubation produced a significant increase in the mean LTP magnitude recorded at CA3-CA1 synapses in hippocampal slices (Fig. 2C) ($149 \pm 10\%$ and $128 \pm 7\%$ of baseline fEPSP slope in simvastatin-treated slices ($n = 9$ slices/9 animals) and vehicle-treated slices ($n = 9$ slices/9 animals), respectively; $P = 0.007$).

Sucrose-based aCSF was used to facilitate the health of brain slices. However, preparing slices in sucrose-based aCSF can limit the magnitude of LTP due to increased synaptic inhibition that occurs as a consequence of the sucrose-mediated neuroprotection of GABAergic interneurons (Kuenzi et al. 2000). Therefore, to rule out the possibility that the increase in LTP magnitude stimulated by simvastatin could be underestimated because of stronger GABAergic inhibition, we repeated the simvastatin incubation experiments in slices prepared using regular aCSF, rather than sucrose-based aCSF. As shown in Fig. 3A and consistent with results obtained in sucrose-prepared slices, simvastatin incubation did not affect the input/output relationship (Fig. 3A) or the PPF ratio (Fig. 3B), but it significantly increased the magnitude of LTP (Fig. 3C) ($148 \pm 7\%$ and $127 \pm 5\%$ of baseline fEPSP slope in simvastatin-treated slices ($n = 11$ slices/8 animals) and vehicle-treated slices ($n = 10$ slices/9 animals), respectively; $P = 0.025$). These experiments demonstrate that simvastatin treatment over a period of 2-4 hours enhances the magnitude of LTP at CA3-CA1 synapses, regardless of slicing conditions.

Acute simvastatin treatment increases the phosphorylation of Akt

To explore the potential molecular mechanisms by which simvastatin modulates synaptic plasticity, we measured the levels of phospho-Akt (p-Akt) and total Akt, which are increased by chronic simvastatin treatment *in vivo* (Li et al., 2006), by immunoblot analyses. The results showed that the levels of p-Akt in the CA1 region were increased significantly (about 2.5 fold, $P < 0.01$) in the hippocampal slices treated with simvastatin ($n=15$ slices/5 mice) for 2 hr, but not for 20 min, compared to slices treated with vehicle ($n=15$ slices/5 mice) (Fig. 4A and 4B). To confirm the upregulation of Akt phosphorylation by simvastatin in the same slices used for LTP recording, we also measured the level of p-Akt in the homogenate of individual hippocampi that had undergone LTP induction (Fig. S1). Consistent with results obtained from non-stimulated slices, the level of p-Akt in simvastatin-treated slices increased by about 2.8 fold compared to vehicle-treated slices ($n = 7$ slices/animals, $P = 0.026$), corresponding with enhanced magnitude of LTP in simvastatin-treated slices ($140 \pm 12\%$) compared to that ($120 \pm 11\%$) in vehicle-treated slices ($n = 7$ slices/animals, $P = 0.047$).

PI3K inhibitor suppresses simvastatin-induced enhancement of LTP

To determine if upregulation of Akt phosphorylation is responsible for the enhancement of LTP by simvastatin treatment, we used a PI3K inhibitor LY294002 that inhibits the phosphorylation of Akt (Sanna et al., 2002, Opazo et al., 2003). We used 20 μ M LY294002 in our experiments, as it has been shown that high concentrations of LY294002 affect basal synaptic transmission (Opazo et al., 2003). Before conducting LTP experiments, immunoblot analysis was performed to confirm that a 40 min incubation in LY294002 at 20 μ M decreases the levels of p-Akt in hippocampal slices. As the results show (Fig. S2), LY294002 partially but significantly decreased the level of p-Akt in simvastatin- and vehicle-treated slices.

Next, we studied the effect of LY294002 on the magnitude of LTP from simvastatin and vehicle-treated slices. In the literature, there are inconsistent results regarding the role of the PI3K/Akt pathway in the induction and expression of LTP at hippocampal CA3-CA1 synapses (Sanna et al., 2002, Opazo et al., 2003). To study the effect of LY294002 on different phases of LTP, we first conducted experiments investigating the effect of LY294002 on LTP expression (already-induced LTP). Hippocampal slices were treated with simvastatin or vehicle as described above for LTP experiments. Twenty minutes after LTP was induced, LY294002 bath application began and continued for the remainder of the experiment. As shown in Fig. 5A, LY294002 application had no differential effect on established LTP in simvastatin or vehicle slices. Although there was a decrement of the LTP magnitude in both simvastatin- and vehicle-treated slices coinciding with the addition of LY294002, the decrement also occurred in vehicle-treated slices in the absence of LY294002, indicating that the decremting LTP magnitude was not caused by the presence of LY294002. The reason for the decrement in the LTP magnitude is not clear but it might be caused by the presence of dimethyl sulfoxide (DMSO), the solvent for LY294002, which was added in the vehicle as a control. The side effects of DMSO on synaptic activity have been reported (Sawada and Sato, 1975, Lu and Mattson, 2001, Tsveytlynska et al., 2005). Thus, when DMSO is used as a solvent in an experiment, proper control experiments are required to control for potential effects of DMSO. While the exact mechanism responsible for the effects of DMSO is beyond the scope of this study, our results show that LY294002 (inhibition of p-Akt) had no significant effect on the expression of LTP.

Next, we investigated the effect of LY294002 on the induction of LTP. Hippocampal slices were subjected to 4 different treatments: vehicle, simvastatin, vehicle+LY294002, and simvastatin+LY294002. LY294002 was bath applied at least 40 min prior to LTP induction and was present throughout the experiment. As shown in Fig. 5B, the LTP magnitude from simvastatin-treated slices was increased significantly compared to that from vehicle-treated slices ($145 \pm 9\%$ vs. $129 \pm 6\%$, simvastatin vs. vehicle, $n = 10$ slices/animals, $P = 0.025$), consistent with data in Fig. 2C and 3C. For slices treated with simvastatin+LY294002, the LTP magnitude was significantly decreased to $119 \pm 7\%$ ($n = 7$ slices/animals, $P = 0.045$ compared with the LTP magnitude from simvastatin slices), indicating that inhibiting phosphorylation of Akt suppressed the enhancement of LTP by simvastatin treatment. Interestingly, for slices treated with vehicle+LY294002 ($n = 10$ slices/animals), the LTP magnitude was unchanged ($129 \pm 7\%$) compared to slices treated with vehicle alone ($129 \pm 6\%$).

DISCUSSION

We have shown recently that chronically administered simvastatin *in vivo* enhances spatial learning and memory in non-transgenic mice as well as in a transgenic mouse model of AD (Li et al., 2006). Our findings corroborate evidence in human epidemiological studies (Jick et al., 2000, Wolozin et al., 2000, Wolozin et al., 2007) and rodent models of cerebral ischemia and/or traumatic brain injury which argue for a positive effect of statins on cognition (Balduini et al., 2003, Chen et al., 2003, Lu et al., 2007a, Wu et al., 2008). However, the cellular and molecular mechanisms underlying the beneficial effects of simvastatin on learning and memory remain elusive. The present study was carried out to investigate the effect of acute *in vitro* simvastatin treatment on LTP at CA3-CA1 synapses, a cellular model of learning and memory, recorded in brain slices from wild type C57BL/6 mice. Our results demonstrate that: 1) Acute simvastatin treatment for up to 4 hrs does not alter basal synaptic transmission or the PPF ratio in hippocampal slices; 2) While a 20-min treatment with simvastatin is not sufficient to affect the magnitude of hippocampal LTP, a prolonged incubation with simvastatin for 2-4 hr significantly enhances the magnitude of LTP in hippocampal slices; 3) Consistent with the results from LTP experiments, simvastatin treatment for 2 hr, but not 20 min, significantly increases the phosphorylation (activation) of Akt in the CA1 region of the hippocampus; and

4) A PI3K inhibitor LY294002, which inhibits the phosphorylation of Akt, suppresses the enhancement of LTP in SV-treated slices.

In a previous study, we have shown that enhancement of learning and memory by chronic simvastatin treatment is associated with an increased level of activated (phosphorylated) Akt (p-Akt) in the brain of wild type mice (Li et al., 2006). In a rat model of TBI, simvastatin treatment activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in hippocampus (Wu et al. 2008), and is responsible for mediating neurorestorative effects of simvastatin. In addition, simvastatin rapidly triggers translocation of Akt to the plasma membrane of cultured endothelial cells (Skaletz-Rorowski et al., 2003). The activation of Akt (i.e. the level of p-Akt) is enhanced as early as 15 minutes after exposure to simvastatin (Kureishi et al., 2000, Skaletz-Rorowski et al., 2003). Another statin, pravastatin, was also found to activate Akt in endothelial cells (Nakao et al., 2007).

Our current understanding of NMDA receptor-dependant LTP divides the phenomenon into “early”, protein-synthesis independent, and “late”, protein-synthesis dependent, phases (Malenka and Bear, 2004). The present study examines early LTP, for which the underlying intracellular signaling cascades have been extensively investigated. The role of the PI3K/Akt pathway in the regulation of LTP is not well understood. While one study using rat hippocampal slices showed that PI3K/Akt activation is required for the expression but not the induction of LTP at CA3-CA1 synapses (Sanna et al., 2002), another study using mouse hippocampal slices demonstrated that PI3K/Akt activation contributes to the induction of LTP rather than the expression or maintenance of LTP at CA3-CA1 synapses (Opazo et al., 2003). The reasons for the discrepancy were not clear but might be attributable to the differences in the concentration of PI3K inhibitors and in the animal species (rat vs. mouse) used in these studies. In the present study, we found that inhibition of Akt phosphorylation by LY294002 had no effect on the expression of hippocampal LTP in either simvastatin or vehicle-treated slices but it significantly suppressed the induction of LTP in simvastatin-treated slices such that the LTP magnitude was similar to that from vehicle-treated slices (Fig. 5). Interestingly, LY294002 did not affect the induction of LTP in vehicle-treated slices under our experimental conditions. It is plausible that, in vehicle-treated slices, the inhibition of basal level of p-Akt by LY294002 was not sufficient to affect the magnitude of LTP, as other LTP-inducing mechanisms exist. However, in simvastatin-treated slices, the level of p-Akt is upregulated, causing a greater contribution of p-Akt to the induction process and enhancing LTP. Thus, inhibition of Akt phosphorylation by LY294002 would produce a significant suppression of LTP in simvastatin-treated slices. Taken together, our data indicate that enhanced Akt phosphorylation is a molecular mechanism contributing to the simvastatin-induced increase in the magnitude of early LTP.

If p-Akt levels can be enhanced in cultured endothelial cells after only 15 minutes of exposure to simvastatin (Kureishi, 2000, 1004), why did the 20-minute exposure in our study not significantly affect the level of p-Akt? We may not be able to compare a monolayer endothelial cell culture with a 400 μ m-thick brain slices. It is likely that a continuously perfused hippocampal slice requires a longer exposure to allow full penetration of simvastatin into the slice and/or sufficient upregulation of the PI3K/Akt pathway. This notion is consistent with our finding that incubation of brain slices with simvastatin for 2 hr indeed increases the level of p-Akt significantly and enhances the magnitude of hippocampal LTP. Therefore, we conclude that hippocampal slices require an exposure to simvastatin greater than 20 min before LTP enhancement can occur under the experimental conditions used in the present study.

While our findings that simvastatin increases hippocampal LTP in C57BL/6 mice parallel the behavioral data that simvastatin enhances hippocampal-dependent learning and memory, these results are seemingly in contrast with reports showing that hippocampal LTP is decreased by

an acute treatment with a different statin, compactin, in mice (Kotti et al., 2006, Kotti et al., 2008). However, we do not feel that these results are necessarily in conflict. The differences in experimental conditions may explain the discrepancy. Firstly, although all statins block HMG-CoA reductase, they possess subtle differences in their chemistry that endow them with different properties (Jones, 2003). While simvastatin was used in our study, compactin was used in the study of Kotti et al. (2006). Compactin is the first statin isolated, but it is not approved for human use because of toxicity concerns (Endo, 2004). Therefore, it is possible that the impact of compactin on neuronal function could be different from that of simvastatin. Secondly, there is a difference in LTP induction protocols. While we used a high-frequency stimulation protocol, Kotti et al. (2006) utilized a theta-burst protocol. It is now appreciated that different stimulation protocols induce LTP with varying molecular mechanistic requirements (Lisman, 2003). Other studies have reported inconsistencies between HFS- vs. theta-burst-induced LTP within a single experimental system (Hoffman et al., 2002, Costa and Grybko, 2005). Thirdly, the age of the animals is different between the two studies. While we used mature (3-4 months old) mice, Kotti et al (2006) used young (4-6 wks old) mice. The molecular mechanisms underlying LTP induction evolve during development (Esteban et al., 2003, Yasuda et al., 2003). C57BL/6 mice younger or older than 2 months have been shown to require different molecular mechanisms for expression of early LTP (Lu et al., 2007b). Therefore, it is possible that modulation of LTP by statin exposure depends upon the developmental state of the animals.

In conclusion, the present study demonstrates that simvastatin can enhance early LTP in hippocampal slices from C57BL/6 mice when administered acutely over 2-4 hours, likely by modulating a signaling pathway involving the activation of Akt. Our findings provide a potential cellular basis for the beneficial effects of simvastatin on cognitive function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

aCSF	artificial cerebral spinal fluid
AD	Alzheimer's disease
Akt	protein kinase B
fEPSPs	field excitatory postsynaptic potentials
HFS	high-frequency stimulation
HMG-CoA	3-hydroxy-3-methylglutaryl CoA reductase
LTP	long-term potentiation
LY	LY294002
NMDA	<i>N</i> -methyl <i>D</i> -aspartate
PI3K	phosphatidylinositol 3-kinase

PPF	paired-pulse facilitation
SV	simvastatin
Veh	vehicle

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Fig. 1. Basal synaptic transmission and early LTP are unaffected by a 20-minute exposure to simvastatin

Vehicle was bath applied to hippocampal slices for the first 10 min of baseline stimulation, and simvastatin (10 μ M) application ensued for the final 20 min of baseline prior to HFS (arrow) and continued for the duration of the experiment. **A:** Paired-pulse facilitation (PPF) ratio was unchanged during simvastatin exposure (1.7 ± 0.05) compared to vehicle (1.7 ± 0.06). **B:** Mean magnitude of LTP from slices treated with simvastatin or vehicle. Mean magnitude of LTP, averaged over the last 5 min of run-out (min 65-70), was unaffected by 20-min simvastatin treatment ($P = 0.583$). LTP from simvastatin-treated slices was $147 \pm 11\%$ ($n = 7$ slices/6 mice), and $137 \pm 12\%$ ($n = 7$ slices/6 mice) from vehicle-treated slices. Also, fEPSP slope did not change after onset of drug application, indicating basal synaptic transmission was unaffected by simvastatin. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from a pair of slices treated with vehicle or simvastatin.

Fig. 2. Prolonged incubation in simvastatin significantly enhances early LTP in slices prepared in sucrose-based aCSF

A: Stimulus response curves for slices prepared in sucrose-based aCSF and incubated in simvastatin or vehicle for 2-4 hours. Incubation in simvastatin ($n = 5$ slices) did not alter the input/output curves over the range of baseline stimulation intensities utilized for baseline compared to vehicle-treated slices ($n = 4$ slices). **B:** Paired-pulse facilitation (PPF) ratio from slices prepared in sucrose-based aCSF and incubated in simvastatin or vehicle. PPF ratio was unchanged during baseline stimulation after incubation in simvastatin (1.6 ± 0.06) compared to vehicle (1.6 ± 0.06). **C:** The mean magnitude of LTP from simvastatin-treated slices ($149 \pm 10\%$, $n = 9$ slices/9 animals) was significantly higher than that from vehicle-treated slices ($128 \pm 7\%$, $n = 9$ slices/9 animals) ($**P = 0.007$). For clarity purposes, error bars are shown in only one direction. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from a pair of slices treated with vehicle or simvastatin.

Fig. 3. Slicing in regular aCSF followed by 2-4 hr incubation in simvastatin significantly enhances early LTP

A: Stimulus response curves for slices prepared in regular aCSF and incubated in simvastatin or vehicle for 2-4 hours. Incubation in simvastatin (18 slices/7 animals) did not alter basal transmission over the range of stimulus intensities utilized for baseline acquisition compared to slices incubated in vehicle (9 slices/5 animals). **B:** Paired-pulse facilitation (PPF) ratio after incubation in simvastatin or vehicle. PPF ratio was not affected by simvastatin incubation.

C: Mean magnitude of LTP from slices incubated in simvastatin or vehicle. The magnitude of LTP from simvastatin-treated slices ($148 \pm 7\%$, $n = 11$ slices/8 animals) was significantly higher ($*P = 0.025$) than that from slices incubated in vehicle ($127 \pm 5\%$, $n = 10$ slices/9 animals). For clarity purposes, error bars are shown in only one direction. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from slices treated with vehicle or simvastatin.

Fig. 4. Effect of simvastatin treatment on the level of p-Akt and total Akt

A: Representative immunoblot images of p-Akt and total Akt in the homogenate of CA1 region of hippocampal slices treated with simvastatin (SV) or vehicle (Veh) for 20 min or 2 hr. **B:** Densitometric analysis of immunoblots (normalized by the amount of tubulin) with the levels in vehicle-treated group set as 100%. Data represent means $\pm$ SEM (n=15 slices/5 mice per treatment). The results showed that the levels of p-Akt in the CA1 region were increased significantly (about 2.5 fold) in the hippocampal slices treated with simvastatin for 2 hr, but not for 20 min, compared to slices treated with vehicle. ** $P = 0.008$.

Fig. 5. PI3K inhibitor LY294002 suppresses simvastatin-induced enhancement of LTP

A. Mean magnitude of LTP from slices incubated in simvastatin or vehicle with LY294002 (LY) (20 μ M) applied 20 min after LTP induction in simvastatin (SV) + LY and vehicle (Veh) + LY slices. The vehicle slices were treated with vehicle (containing 0.1% DMSO) with no application of LY and served as control. The magnitude of LTP from simvastatin + LY slices ($141 \pm 9\%$, $n = 8$ slices/8 animals) was significantly higher ($*P = 0.038$) than that from vehicle + LY slices ($116 \pm 4\%$, $n = 8$ slices/8 animals). The magnitude of LTP from vehicle + DMSO was $118 \pm 6\%$ ($n = 6$ slices/6 animals) similar to that from vehicle + LY slices. The results show LY does not affect the expression of LTP in either simvastatin-or vehicle-treated slices.

B. Mean magnitude of LTP from slices incubated in simvastatin or vehicle and treated with LY294002 (20 μ M) or DMSO for at least 40 min pre-induction and for the rest of the experiment. The LTP magnitude from simvastatin-treated slices was increased significantly compared to that from vehicle-treated slices ($145 \pm 9\%$ vs. $129 \pm 6\%$, $n = 10$ slices/animals, $P = 0.025$). From the slices treated with simvastatin + LY, the LTP magnitude was significantly decreased to $119 \pm 7\%$ ($n = 7$ slices/animals, $*P = 0.045$ compared with the LTP magnitude from simvastatin-treated slices). From slices treated with vehicle + LY ($n = 10$ slices/animals), the LTP magnitude was unchanged ($129 \pm 7\%$) compared to slices treated with vehicle + DMSO ($129 \pm 6\%$). For clarity purposes, error bars are shown in only one direction. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from slices in different treatment groups.