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SIMVASTATIN-MEDIATED ENHANCEMENT OF LONG-TERM POTENTIATION IS DRIVEN BY FARNESYL-PYROPHOSPHATE DEPLETION AND INHIBITION OF FARNESYLATION

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Abstract—Simvastatin (SV), a competitive inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase and a widely prescribed treatment for hypercholesterolemia, exerts numerous positive pleiotropic effects that are thought to occur independent of its cholesterol-lowering properties. In previously published work, we have shown that chronic SV treatment rescues cognitive function in a transgenic mouse model of Alzheimer's disease, and enhances learning and memory in non-transgenic mice without affecting total brain cholesterol and amyloid-beta levels. More recently, we demonstrated the ability of SV to enhance long-term potentiation (LTP) in the CA1 region of the hippocampus in slices from wild-type C57BL/6 mice via a mechanism dependent upon phosphatidylinositol 3-kinase (PI3-K)/Akt activation during LTP induction. The present study was conducted to better understand the molecular mechanisms underlying SV-induced enhancement of LTP. Specifically, it was found that inhibiting production of isoprenoid intermediates in the biosynthetic pathway for cholesterol triggers the downstream events leading to enhanced LTP. Interestingly, two major isoprenoid intermediates exhibit differential effects. Replenishment of farnesyl pyrophosphate, but not geranylgeranyl pyrophosphate, abolished the LTP-enhancing ability of SV. In parallel to this finding, inhibiting farnesylation, but not geranylgeranylation, replicated the enhancement of LTP caused by SV. Finally, inhibiting farnesylation promotes the activation of Akt during the induction phase. Together, these results suggest that SV enhances LTP in CA1 by modulating isoprenylation-dependent molecular pathways downstream of farnesyl transferase. These findings will aid in the identification of novel therapeutic targets that modulate synaptic and cognitive function.

Key words: statins, isoprenoids, protein prenylation, synaptic plasticity, hippocampus, long-term potentiation.

Statins directly inhibit the rate-limiting enzyme of the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which converts HMG-CoA to mevalonate (Endo, 2004). Inhibiting this reaction not only reduces *de novo* cholesterol biosynthesis, but also reduces production of non-sterol intermediates, known as isoprenoids, downstream of mevalonate and preceding cholesterol. Isoprenoids, such as farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP), serve as lipid attachments for all members of the small GTPase superfamilies, which include the well-known Ras, Rho, and Rac (McTaggart, 2006). The isoprenylation state of GTPases alters their intracellular trafficking, subcellular localization, and interactions with substrates and, therefore, modifies their function and functions of downstream effectors (McTaggart, 2006). Consequently, reducing isoprenoid availability or the process of isoprenylation itself can affect a diverse group of intracellular signaling pathways and processes. Some lipophilic statins, such as simvastatin and lovastatin, are capable of crossing the blood–brain barrier (Jones, 2003). The effects of statins are diverse and extend across several disciplines. Thus, it is imperative to understand how statins affect cognitive function, and a number of experimental and epidemiological studies have been conducted to this end (Cole and Vassar, 2006).

Statins have been shown, to some extent, to be therapeutic and neuroprotective in humans, though these results are not without controversy. Some epidemiological studies indicate a reduced prevalence of Alzheimer's disease (AD) or dementia in statin-prescribed populations (Jick et al., 2000; Wolozin et al., 2000). However, there are conflicting reports that statins are not neuroprotective (Collins et al., 2002; Shepherd et al., 2002), and some statin users suffer memory loss that is ameliorated by withdrawal from statin treatment (Wagstaff et al., 2003). In support of a neuroprotective role for statins, it has been observed that statins reduce pro-inflammatory responses of microglia after amyloid-β peptide exposure *in vitro* (Cordle and Landreth, 2005) and *in vivo* (Clarke et al., 2008), and protect cultured cortical neurons from excitotoxicity after exposure to *N*-methyl D-aspartate (NMDA) (Zacco et al., 2003) and monosodium glutamate (Bösel et al., 2005). Also, a recent

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Abbreviations: aCSF, artificial cerebral spinal fluid; AD, Alzheimer's disease; Akt, protein kinase B; fEPSPs, field excitatory postsynaptic potentials; FPP, farnesyl pyrophosphate; FTase, farnesyl transferase; FTI, farnesyl transferase inhibitor; GGPP, geranylgeranylpyrophosphate; GGTase, geranylgeranyl transferase; GGTI, geranylgeranyl transferase inhibitor; HFS, high-frequency stimulation; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LTP, long-term potentiation; Mev, mevalonate; NMDA, *N*-methyl D-aspartate; PI3-K, phosphatidylinositol 3-kinase; PPF, paired-pulse facilitation; SV, simvastatin; Veh, vehicle.

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study found elevated levels of FPP and GGPP in the brains of AD patients suggesting that reducing isoprenoid production may prove therapeutic (Eckert et al., 2009). Indeed, in previously published work from our laboratory, a simvastatin (SV)-supplemented diet rescued learning and memory in a transgenic mouse model of AD independent of changes in amyloid beta pathology (Li et al., 2006). Interestingly, dramatic memory improvements are also observed in non-transgenic wild-type (WT) littermate controls. A similar effect has been observed in adult rats administered SV for 25 days prior to testing in passive avoidance or object-in-place tasks (Douma et al., 2011). It is therefore possible that SV can augment the processes underlying learning and memory in normal, non-diseased brains. Chronic statin treatment stimulates production of brain-derived neurotrophic factor (BDNF) (Wu et al., 2008), increases levels of NMDA receptors (Wang et al., 2009), promotes neurogenesis, and increases cerebral blood flow (Chen et al., 2003). Additionally, we recently reported that treatment of hippocampal slices for several hours with SV increases the magnitude of NMDA receptor-dependent long-term potentiation (LTP), a mechanism thought to mediate memory at the cellular level, in the CA1 region in the brains of young adult C57BL/6 mice (Mans et al., 2010). Many of the pleiotropic effects described above have been attributed to reduced isoprenoid production and subsequent altered small GTPase function.

In the current study, we investigate the mechanism by which SV augments LTP in the CA1 region of the hippocampus of C57BL/6 mice. We test the hypothesis that SV-induced reduction of isoprenoid production leads to enhanced LTP. We also test the related hypothesis that inhibition of isoprenylation contributes to the SV-induced LTP enhancement we observe. In support of these hypotheses, evidence is presented demonstrating that replenishing FPP, but not GGPP, abolishes SV-induced LTP enhancement. Furthermore, we find that inhibiting farnesylation, but not geranylgeranylation, mimics the LTP-enhancing property of SV. Lastly, we present data suggesting that inhibiting farnesylation augments the recruitment of phosphatidylinositol 3-kinase (PI3-K) activity during LTP induction.

EXPERIMENTAL PROCEDURES

Animals

Three- to four-month-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA; 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-h 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.

Slice preparation and electrophysiology

Hippocampal slices (400 μM) were prepared from male C57BL/6 mice using methods described previously (Mans et al., 2010) with modifications. Briefly, mice were anesthetized with isoflurane and decapitated. Their brains were removed and immersed in ice-cold "high-sucrose" artificial cerebral spinal fluid (aCSF) composed of (in mM): NaCl 85; KCl 2.5; $MgSO_4$ 4; $CaCl_2$ 0.5; NaH_2PO_4 1.25; $NaHCO_3$ 25; glucose 25; sucrose 75; 290–300 mOsm. This solution contains less Na^+ and Ca^{2+} and higher Mg^{2+} than sodium-based aCSF and promotes neuronal survival during the slicing procedure by reducing excitotoxicity (Kuenzi et al., 2000). Coronal slices of dorsal hippocampi were cut on a vibratome (Leica) and incubated in high-sucrose aCSF for 10 min, then for $\geq$1 h in regular aCSF containing (in mM): NaCl 119; KCl 2.5; $CaCl_2$ 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1; $NaHCO_3$ 26; and glucose 10 saturated with 95% O_2–5% CO_2 (pH 7.4). To record field excitatory postsynaptic potentials (fEPSPs), slices were placed in a submersion recording chamber and continuously perfused at approximately 1.5–2.0 ml/min with aCSF warmed to 26–28 °C and recirculated via peristaltic perfusion pump. CA1 extracellular dendritic fEPSPs were recorded (Axopatch 200B, Molecular Devices, Sunnyvale, CA, USA) using standard methods (Mans et al., 2010). 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 15 min, NMDA receptor-dependent LTP was induced with a high-frequency stimulation (HFS) protocol (four 0.5-s trains of 100 Hz stimulation applied at 20 s intervals) (Mans et al., 2010). 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 baseline intensity immediately after HFS.

Preparation of solutions for electrophysiology experiments

Simvastatin (SV). Simvastatin was purchased from Calbiochem (Cat#567020). Prior to its use in experiments, SV was converted from its inactive lactone prodrug form to its active dihydroxy open acid form by alkaline hydrolysis as described previously (Mans et al., 2010) (first dissolving 50 mg of the compound in 1 ml of ethanol (100%) and then adding 0.813 ml of l N NaOH). This stock solution was stored in aliquots at −20 °C (for up to 1 month). On the day of use, the SV stock solution was neutralized with l N HCl to pH of 7.4 and diluted in aCSF. The final concentration of SV in the recording solution was 10 μM. In addition, a vehicle solution lacking SV was added to aCSF to serve as control.

Mevalonate (Mev). Mevalonate was purchased from Sigma (Cat # M-4667) and prepared according to previously published methods (Essig et al., 1998; Wagner et al., 2000). Briefly, mevalonate was dissolved before undergoing alkaline hydrolysis in 0.1N NaOH (heated at 50 °C for 2 h), and 0.2 M stock solutions (pH 7.0) were stored at −20 °C.

Farnesol (FOH). *Trans*, *trans*-farnesol (96%) was purchased from Sigma (Cat# 27754). To prepare 0.2 mM FOH, 1.85 μl of farnesol was initially pipetted into 4 μl of ethanol to improve solubility. This solution was then diluted into 40 ml of aCSF to reach a final concentration of 0.2 mM. 0.01% Ethanol was used for vehicle control.

Geranylgeraniol (GGOH). Geranylgeraniol ($\geq$85%) was purchased from Sigma (Cat# G3278). To prepare 0.2 mM GGOH, 2.42 μl of GGOH was pipetted into 4 μl of ethanol to improve solubility. This solution was then diluted into 40 ml of aCSF to reach a final concentration of 0.2 mM. Control solutions contained 0.01% ethanol.

Farnesyl transferase inhibitor (FTI). FTI-277 was purchased from Calbiochem (Cat# 344555). A 1 mM stock solution