Neuroscience 329 (2016) 264–274

SIMVASTATIN INHIBITS PROTEIN ISOPRENYLATION IN THE BRAIN

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Abstract—Evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, may reduce the risk of Alzheimer's disease (AD). Statin action in patients with AD, as in those with heart disease, is likely to be at least partly independent of the effects of statins on cholesterol. Statins can alter cellular signaling and protein trafficking through inhibition of isoprenylation of Rho, Cdc42, and Rab family GTPases. The effects of statins on protein isoprenylation *in vivo*, particularly in the central nervous system, are poorly studied. We utilized two-dimensional gel electrophoresis approaches to directly monitor the levels of isoprenylated and non-isoprenylated forms of Rho and Rab family GTPases. We report that simvastatin significantly inhibits RhoA and Rab4, and Rab6 isoprenylation at doses as low as 50 nM *in vitro*. We also provide the first *in vivo* evidence that statins inhibit the isoprenylation of RhoA in the brains of rats and RhoA, Cdc42, and H-Ras in the brains of mice treated with clinically relevant doses of simvastatin.

Key words: Alzheimer's disease, isoprenylation, simvastatin, RhoA, Cdc42, Rab.

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Abbreviations: 2D, two-dimensional; AD, Alzheimer's disease; APP, amyloid precursor protein; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GGT, geranylgeranyl transferase; GGTI, GGT inhibitor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; Icmt, isoprenylcysteine-O-c transferase; pI, isoelectric point; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

http://dx.doi.org/10.1016/j.neuroscience.2016.04.053

INTRODUCTION

Statins are widely prescribed drugs for the treatment of hypercholesterolemia, acting to reduce plasma cholesterol levels by inhibiting the rate-limiting enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-met hylglutaryl-coenzyme A (HMG-CoA) reductase (Endo, 1992). Many retrospective studies have suggested that patients treated with statins have up to a 70% reduced risk of developing Alzheimer's disease (AD) (Wolozin et al., 2006), though evidence from randomized control trials suggests that statins might not be sufficient to prevent progression of AD (Feldman et al., 2010; Sano et al., 2011). The link between cholesterol levels and AD is mixed, with most studies showing no correlation between cholesterol levels and AD (Wood et al., 2005). Although several studies support a link between high midlife cholesterol and decreased risk of developing AD (Kivipelto et al., 2001; Solomon et al., 2009), other studies have not confirmed this linkage (Kalmijn et al., 2000; Tan et al., 2003). However, high cholesterol in late life has been shown to reduce the risk of developing AD (Mielke et al., 2005). As low cholesterol is not clearly linked to decreased AD risk, these data suggest that statins may act, at least in part, independently of cholesterol to reduce AD risk. Cholesterol-independent effects of statins are of broad relevance, since the therapeutic benefit of these drugs in cardiovascular disease and stroke are thought to be at least partially independent of their effects on cholesterol (Pezzini et al., 2009; Zhou and Liao, 2009).

Statins may exert their pleiotropic effects through reduction of isoprenoid intermediates in the cholesterol biosynthetic pathway, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Liao and Laufs, 2005). FPP and GGPP are 15- and 20-carbon chains, respectively, that are covalently linked to the C-termini and required for the membrane localization and function of the Ras superfamily of small G-proteins, including Rho- and Rab-family proteins. Rho-family proteins, such as Rho, Rac and Cdc42, were initially identified as key regulators of the actin cytoskeleton (Nobes and Hall, 1995), and also have been shown to regulate numerous intracellular signaling pathways, including amyloid precursor protein (APP) processing (Zhou et al., 2003; Ostrowski et al., 2007). Rab-family proteins are known to be critical mediators of intracellular vesicular trafficking, including transport of APP (Dugan et al., 1995; McConlogue et al., 1996). It recently has been reported that statins decrease the levels of FPP and GGPP in the rodent brain, and that FPP and GGPP are upregulated in aged mice (Hooff et al., 2012) and in

post-mortem brain tissue from AD patients (Eckert et al., 2009), suggesting that alterations in protein prenylation may play a role in neuronal aging and neurodegeneration.

Rho- and Rab-family proteins are modified primarily by geranylgeranylation, and the modification is site specific. Rho proteins carry a C-terminal CaaX isoprenylation motif, and isoprenylation occurs on this C-terminal cysteine (Kinsella et al., 1991). Rab-family proteins carry either a CXC or CC C-terminal isoprenylation motif, and both C-terminal cysteines are isoprenylated (Khosravi-Far et al., 1991). Rho-family proteins are further processed by the prenyl protease, Rce1, to remove-aaX residues (Boyartchuk et al., 1997), and the cysteine is then carboxymethylated by the enzyme isoprenylcysteine-O-c transferase (Icmt) (Dai et al., 1998). CXC Rab proteins are not cleaved by Rce1, but CXC Rab proteins are carboxymethylated by Icmt (Bergo et al., 2001). CC Rab proteins are not methylated, likely because the steric hindrance of adjacent cysteines does not allow access to the Icmt enzyme (Smeland et al., 1994). The carboxymethylation of GTPases is functionally important for GTPase function (Papaharalambus et al., 2005; Leung et al., 2006).

While numerous *in vitro* studies have demonstrated isoprenoid-dependent effects of statins in neurons and glial cells (Jiang et al., 2004; Pooler et al., 2006; Kim et al., 2009), it remains unclear whether statins reach sufficient concentrations in the brain to affect isoprenylation in the central nervous system. It has been demonstrated that statins pass the blood–brain barrier, and in mice simvastatin reaches peak concentrations of 600 nM in the brain (Johnson-Anuna et al., 2005). While it has been reported that statins inhibit membrane localization of Rho and Rab proteins in cultured cells at doses as low as 200 nM (Ostrowski et al., 2007), it is unknown whether statins persist in the brain at sufficient concentrations to inhibit isoprenylation. In addition, no studies have examined whether statin treatment alters membrane localization or isoprenylation of Rho or Rab GTPases *in vivo* in the brain.

To directly study the effects of statins on protein isoprenylation, we developed a two-dimensional (2D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) approach, similar to that previously used to demonstrate that statins inhibit protein isoprenylation in peripheral mononuclear cells (Cicha et al., 2004). The carboxymethylation of the Rho-family proteins, such as RhoA, neutralizes the negative charge of the carboxyl-terminal carboxyl group, resulting in a protein that has a more basic isoelectric point (pI) than the non-carboxymethylated protein, and the carboxymethylated and non-carboxymethylated forms of these proteins may be resolved by isoelectric focusing (Backlund, 1997). As statin inhibition of protein isoprenylation prevents the carboxymethylation step, protein pI serves as a sensitive and direct marker for protein isoprenylation (Cicha et al., 2004). We have demonstrated that 2D SDS–PAGE can be used to quantitate the prenylation status of Rho- and Rab-family proteins *in vitro* in mouse neuro-2a neuroblastoma (N2a) cells. We found that in N2a cells the isoprenylation of Rho and Rab family proteins is inhibited by high doses of simvastatin, and isoprenylation of RhoA and Cdc42 is inhibited at clinically relevant doses of simvastatin as low as 50 nM. Finally, we report the first evidence that simvastatin measurably inhibits protein isoprenylation *in vivo* in the brain. Taken together, these data suggest that the inhibition of protein isoprenylation in the central nervous system can occur following systemic administration of statins, and this finding is of interest as statins are being revaluated for potential benefits in neurological disorders apart from Alzheimer's, such as autism and epilepsy (Ghanizadeh, 2011; Buchovecky et al., 2013; Hagerman and Polussa, 2015).

EXPERIMENTAL PROCEDURES

Materials and reagents

Simvastatin was purchased from Calbiochem (La Jolla, CA, USA) and prepared following the manufacturer's instructions. Simvastatin was converted to the active form by dissolving in absolute EtOH, followed by the addition of 1 M NaOH to a final concentration of 60 mM. This solution was stored at −20 °C until use. Immediately before use, the simvastatin solution was neutralized with 1 M HCl and diluted in vehicle (50% EtOH, 5 mM HEPES, pH 7.2). Cysmethynil was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Precast gels and IPTG pI strips were purchased from Bio-Rad Laboratories (Hercules, CA, USA). An antibody to Rab4 was obtained from Upstate Biotechnology (Waltham, MA, USA). Antibodies to Cdc42, Rab11, Rab1b, Rab5b, RhoA, H-Ras, and Rab6 were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA), and anti-farnesyl from Biorbyt (Cambridge, UK). Additional antibodies to RhoA were purchased from Cytoskeleton (Denver, CO, USA) and Origene (Rockville, MD, USA). Peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (UK). Cell-culture reagents were purchased from Invitrogen™ Life Technologies (Carlsbad, CA, USA).

Cell culture

Mouse N2a cells were obtained from American Type Culture Collection (Manassas, VA, USA). N2a cells were cultured in DMEM with 5% heat-inactivated FBS (Hyclone, Logan, UT), and 1% penicillin/streptomycin.

Treatment of rats with simvastatin and rosuvastatin

Spontaneously hypertensive stroke-prone rats were treated with vehicle, rosuvastatin, or simvastatin (10 mg/kg/day) for 30 days as previously described (Sironi et al., 2005). Briefly, rosuvastatin was dissolved in a small amount of 1% NaCl drinking water. After consumption of this amount, animals had free access to 1% NaCl water. Simvastatin was given by oral gavage in small amounts of 5% carboxymethyl cellulose vehicle. Frozen whole-brain samples were homogenized in lysis buffer (0.5% SDS, 25 mM Tris pH 8.5, 2.5 mM $MgCl_2$), and protein samples were processed and examined for 2D SDS–PAGE and Western blotting as described.