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Simvastatin inhibits protein isoprenylation in the brain

Stephen M. Ostrowski¹, Kachael Johnson^{2,3}, Matthew Siefert³, Sam Shank⁴, Luigi Sironi⁵, Benjamin Wolozin⁶, Gary E. Landreth¹, and Assem G. Ziady^{2,3,4}

¹Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio, USA

²Department of Pediatrics, Emory University, Atlanta, Georgia, USA

³Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

⁴Department of Pediatrics, Case Western Reserve University, Cleveland, Ohio, USA

⁵Department of Pharmacological and Biomolecular Sciences, University of Milan, and Centro Cardiologico Monzino, Milan, Italy

⁶Departments of Pharmacology and Neurology, Boston University School of Medicine, Boston, Massachusetts, USA

⁷Children's Healthcare of Atlanta, Atlanta, Georgia, USA

Abstract

Evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, may reduce the risk of Alzheimer's disease. Statin action in patients with Alzheimer's disease, 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 inhibit 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.

Keywords

Alzheimer's disease; isoprenylation; simvastatin; RhoA; Cdc42; Rab

To whom correspondence should be addressed: Dr. Assem Ziady, Ph.D., Department of Pediatrics, Cincinnati Children's Hospital, Division of Pulmonary Medicine, 3333 Burnet Avenue, MLC 2021, Cincinnati, OH 45229, T: 513-803-9094; F: 513-803-4820 assem.ziady@cchmc.org.

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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-methylglutaryl-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). Rab-CXC 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, Jr., 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 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 reevaluated for potential benefits in neurological disorders apart from Alzheimer's, such as autism and epilepsy (Hagerman and Polussa, 2015; Ghanizadeh, 2011; Buchovecky et al., 2013).

MATERIALS AND METHODS

Materials and reagents

Simvastatin was purchased from Calbiochem (La Jolla, CA) and prepared following the manufacturer's instructions. Simvastatin was converted to the active form by dissolving in absolute EtOH, followed by the addition of 1M 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 1M HCl and diluted in vehicle (50% EtOH, 5 mM HEPES, pH 7.2). Cysmethynil was purchased from Cayman Chemical Co. (Ann Arbor, MI). Precast gels and

IPTG pI strips were purchased from Bio-Rad Laboratories (Hercules, CA). An antibody to Rab4 was obtained from Upstate Biotechnology (Waltham, MA). Antibodies to Cdc42, Rab11, Rab1b, Rab5b, RhoA, H-Ras, and Rab6 were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX), and anti-farnesyl from Biorbyt (Cambridge, UK). Additional antibodies to RhoA were purchased from Cytoskeleton (Denver, CO) and Origene (Rockville, MD). Peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (UK). Cell-culture reagents were purchased from Invitrogen™ Life Technologies (Carlsbad, CA).

Cell culture

Mouse N2a cells were obtained from American Type Culture Collection (Manassas, VA). 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 MgCl₂), and protein samples were processed and examined for 2D SDS-PAGE and Western blotting as described.

Treatment of mice with simvastatin

Chow was compounded by Research Diets, Inc. (New Brunswick, NJ) to deliver 10 mg/kg/day of simvastatin to C57BL/6 mice (n=8) for 30 days (60 mg simvastatin/kg chow). Normal chow was used for C57BL/6 control mice (n=10). After 30 days, mice were sacrificed and brains were excised and homogenized in protease lysis buffer containing 40 µg/ml of esterase inhibitor, ebec lactone B. Lysates were prepared as described for Western blotting.

Protein extraction, isoelectric focusing, and Western blotting

For *in vitro* 2D-SDS PAGE studies, cells were collected and lysed in lysis buffer, with protease and esterase inhibitor (40 µg/ml). Samples were incubated for 5 min at 4°C, heated at 95 °C for 5 min with light vortexing, and cooled to room temperature. Bovine pancreatic RNase and DNase (100 ng/ml) were added for 15 min at room temperature. Protein concentrations were measured by the BCA microplate assay. Lysates were precipitated overnight with 5× volume of ice-cold acetone. For *in vivo* 2D SDS PAGE studies, brain homogenates were prepared by sonication in lysis buffer, followed by precipitation in acetone. Lysates were resuspended in IPG solubilization buffer (7M urea, 2 M thiourea, 1% DTT, 1% CHAPS, 1% carrier ampholytes pI 5–8) to a concentration of 5 mg/ml.

Isoelectric focusing was conducted as previously described (Chen et al., 2008; Ziady and Kinter, 2009) with some variation. From each sample, 200 µl was rehydrated overnight on pI

4–7 or 3–10 IPG strips (Bio-Rad, Richmond, CA), and then focused for 32,000–40,000 volt hours (Bio-Rad Protean IEF cell). Focused strips were subjected to alkylation and reduction. For one-dimensional (1D) SDS-PAGE, cells were harvested and lysed in RIPA buffer (Santa Cruz Biotechnologies, Dallas, TX), DNA was sheared by sonication, and the lysate was centrifuged for 15 min at 14,000 rpm. Protein was quantified by BCA assay, followed by addition of Laemmli sample buffer and boiling under reducing conditions.

Focused strips (2D-SDS PAGE) or 10–20 µg of each protein sample (1D SDS PAGE) were subject to electrophoretic molecular weight separation in the second dimension on precast Criterion 12% gels (Bio-Rad) and then transferred to polyvinylidene fluoride or nitrocellulose membranes. After blocking in Membrane Blocking Solution (Invitrogen), blots were incubated overnight at 4°C with the appropriate primary antibodies at concentrations of 1:200–1:1000. Bands were visualized by incubation of blots with anti-mouse, -rabbit or -goat HRP-conjugated secondary antibodies (1:10,000; 90 min at room temperature) and visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and femto-chemiluminescence reagents (Thermo Fisher Scientific, Rockford, IL).

Western blots of 2D gels were scanned using QuantityOne™ software on the Bio-Rad GS-800 Calibrated Densitometer. A 3–10 pI grid was created using PDQuest™ software and used to measure the bands of interest at the acidic and the basic ends. The average pI of the control group was compared to the treatment group; acidic end to acidic end and basic end to basic end, respectively.

Data quantifications and statistical analysis

Western blots of 2D gels were scanned using the QuantityOne™ software package with a Bio-Rad GS-800 Calibrated Densitometer. Once gels were oriented and aligned, band densities were analyzed using the PDQuest™ 2D-gel analysis software. Ratios of prenylated to total RhoA for each animal were calculated. An unpaired *t*-test or ANOVA, as indicated, was then performed using these values to calculate significance (*P* values).

RESULTS

Simvastatin causes isoelectric 2D-gel shift of CAAX Rho-family proteins

Carboxymethylation of the C-terminal cysteine in the CAAX motif alters the pI of Rho family proteins such as RhoA and Cdc42 with respect to their non-methylated forms. Thus, 2D SDS-PAGE can be used to resolve the methylated from non-methylated species of these proteins. It has been demonstrated previously that for RhoA isolated from the macrophage line, RAW 264.7, the methylated form of the protein has a pI of 6.35 and the non-methylated form has a pI of 5.9 (Backlund, Jr., 1997).

Total cell proteins isolated from untreated N2a cells were isoelectrically focused, subjected to SDS PAGE, then transferred and blotted for RhoA. We found that RhoA migrated as two bands, the first and more dominant representing methylated RhoA at a pI of approximately 6.2–6.6 and the second representing the non-methylated form at a pI of approximately 5.8–6.2 (Figure 1A).

Statin treatment, which inhibits GTPase isoprenylation, has been found to also inhibit the subsequent processing step of GTPase carboxymethylation (Haklai and Kloog, 1991; Law et al., 1992; Lerner et al., 1992). This shifts the pI of RhoA from the basic (methylated) to the more acidic (non-methylated) band on a 2D SDS-PAGE gel (Cicha et al., 2004). To test this effect of statins in our models, we treated N2a cells with 10 μ M simvastatin for 24 h, followed by 2D electrophoresis and Western blotting against RhoA. Lysates from cells treated with this maximal dose of simvastatin exhibited a significant acidic isoelectric shift of RhoA, with the majority of the protein shifted to the band corresponding to the non-carboxymethylated form (basic/acidic = 0.083 ± 0.03 , Figure 1A). No such shift was exhibited in vehicle-treated cells (basic/acidic = 11.13 ± 1.59 , Figure 1A). The altered electrophoretic mobility of RhoA was confirmed by using three different RhoA-specific antibodies (data not shown).

Total cell lysates were then subjected to 2D gel electrophoresis followed by Western blotting using antibody specific for Cdc42. We found that Cdc42 migrated as two bands, the first and more dominant representing methylated Cdc42 that migrated at a pI of approximately 6.4–6.8 and the second representing the non-methylated form at a pI of approximately 6.0–6.4 (Figure 1B). Similar to the results with RhoA, we find that 10 μ M simvastatin treatment led to a shift of the majority of the Cdc42 protein to the band corresponding to the non-carboxymethylated form (basic/acidic = 0.066 ± 0.03 , Figure 1B). We also observed a slightly lower molecular weight band for both RhoA and Cdc42 on the majority of our 2D gel blots. Similar lower molecular weight bands that are not observed on 1D gels have been described for 2D gels of other Ras family (Gromov and Celis, 1998; Gromov et al., 1996).

To test whether these changes were related to changes in protein expression or stability, we examined the total protein levels of RhoA protein after high-dose simvastatin treatment. The levels of RhoA protein were moderately increased (~3 fold), while protein levels of Cdc42 were markedly increased (~5.5 fold), after treatment with 10 μ M of simvastatin (Figure 1A and 1B, right panels), corroborating to our previously published results (Ostrowski et al., 2007). Thus, at high doses of simvastatin it is possible that an increase in steady-state levels of RhoA and Cdc42 may contribute to the observed increased ratio of non-prenylated to prenylated RhoA and Cdc42. Although dose response studies below (Figure 3) with lower doses of simvastatin do not support that a significant portion of non-prenylated protein is due to high dose simvastatin.

Simvastatin causes isoelectric shift of CXC, but not CC Rab proteins

Unlike Rho proteins, Rab proteins do not contain a –CaaX isoprenylation motif, but instead contain a CXC or CC isoprenylation motif. CXC proteins have been shown to be carboxymethylated on both cysteines, while CC proteins are not carboxymethylated (Smeland et al., 1994). 2D SDS-PAGE previously has not been used to examine the effects of statins on the isoprenylation of Rab-family proteins. We predicted that while statins have been shown to block the isoprenylation of both CXC and CC Rab proteins (Ostrowski et al., 2007; Sakamoto et al., 2011); the isoelectric mobility of only CXC, but not CC proteins, would be affected. When lysates of vehicle-treated N2a cells were analyzed by 2D SDS-PAGE, CXC Rab proteins Rab4 and Rab6 migrated predominately as two bands, with the

predominant band representing the methylated form and the lesser band the non-methylated form (Figure 2 A,B). The CC Rab proteins Rab1b and Rab11 migrated only as one band, as absence of carboxymethylation of the c-terminal cysteine precludes electrophoretic mobility changes (Figure 2C).

To test the effect of statins, we treated N2a cells with simvastatin for 24 h, then performed 2D SDS-PAGE followed by western blotting using antibodies specific to the CXC proteins, Rab6 and Rab4 or the CC proteins, Rab1b and Rab11. Statin treatment resulted in acidic shifts in the pI of Rab6 (basic/acidic = 0.52 ± 0.27 , Figure 2A) and Rab4 (basic/acidic = 0.21 ± 0.03 , Figure 2B), but had no effect on the migration of Rab11 (Figure 2C) or Rab1b (data not shown). Of note, unlike CaaX proteins which migrated primarily in two distinct bands for the modified and non-modified forms following statin treatment, the non-modified forms of the CXC Rab proteins, such as Rab4 migrated as a series of bands, possibly due to the fact that these proteins have two isoprenylation sites leading to multiple possible combinations of isoprenylation status for these proteins. In addition, Rab4 and Rab6 previously have been shown to exhibit a small increase in molecular weight in the non-isoprenylated form (Ostrowski et al., 2007), and this was reflected on the 2D SDS-PAGE gels.

Simvastatin inhibits protein isoprenylation at physiologically relevant doses

The isoprenylation-dependent effects of statins have been extensively studied in *in vitro* models. Most studies utilize supraphysiologic doses of statins, and the dose-dependent effects of statins on protein isoprenylation have not been well studied. We used 2D gel analysis to examine the dose-dependent effects of statins on protein isoprenylation. We found that 10 μ M simvastatin was sufficient to shift the vast majority of cellular RhoA and Cdc42 to the non-modified form (Figure 1). Surprisingly, even at doses as low as 500 nM, the majority of cellular RhoA (basic/acidic = 0.18 ± 0.06 , Figure 3A) and Cdc42 (basic/acidic = 0.097 ± 0.02 , Figure 3B) were shifted to the non-modified species. Significantly, we found that at doses as low as 50 nM, ~40–50% of cellular RhoA (basic/acidic = 1.25 ± 0.41) and Cdc42 (basic/acidic = 1.17 ± 0.10) were shifted to the non-modified form. To ensure that these changes were not related to changes in protein expression or stability, we examined the total protein levels of RhoA protein after low-dose simvastatin treatment. The levels of RhoA and Cdc42 protein were not significantly increased in the 10 nM–100 nM dose range, but levels were increased at doses of 200–500 nM, for both proteins (Figure 3A and B 1D gel quantitation). These data suggest that at physiologically relevant doses of simvastatin, the changes in RhoA and Cdc42 isoelectric mobility are directly related to inhibition of protein isoprenylation and are not confounded by effects on protein production or stability.

Simvastatin mediates gel shifts through inhibition of protein isoprenylation

Statins have been shown to have a non-significant effect on cellular cholesterol levels when cells are grown in serum-containing media (Cordle and Landreth, 2005). Therefore, the effects of statins on pI were not a result of changes in cellular cholesterol. To confirm that the changes in pI after simvastatin treatment were mediated through inhibition of protein isoprenylation, we tested whether they could be reversed through repletion of isoprenoid-

pathway intermediates. N2a cells were treated with simvastatin in the presence or absence of mevalonate, GGPP, or FPP. Simvastatin-induced changes in RhoA pI were completely reversed by addition of mevalonate (Figure 4C), indicating that the effects of statins were HMG-CoA reductase dependent and due to reduction of isoprenoid intermediates. Supplementation with GGPP also reversed the RhoA isoelectric shift (Figure 4D, quantitation plot), while supplementation with FPP did not reverse any of the effects of simvastatin (Figure 4E). These experiments strongly suggest that the effects of statins on the pI of RhoA were specifically mediated by decreasing the pools of GGPP and blocking protein isoprenylation. While there is substantially more GGPP than FPP in the brain (Eckert et al., 2009), we chose concentrations of GGPP and FPP similar to those that previously have been shown to reverse farnesylation-dependent (Dai et al., 2007) and geranylgeranylation-dependent (Cordle and Landreth, 2005) effects of statins in cell-culture models. When Cysmethynil, an inhibitor of isoprenylcysteine carboxyl methyltransferase (Icmt), we observed a significant portion of RhoA ($28.04 \pm 9.56\%$) shift towards the acidic pI (Figure 4F). Icmt mediates the methylation of the carboxy-terminus, the final step of prenylation for CaaX motif containing proteins. Therefore, these data further demonstrate that carboxymethylation is a significant determinant of the pI shifts observed for prenylated CaaX motif-containing proteins.

To further examine whether changes in isoelectric point are mediated through inhibition of protein isoprenylation, we employed a direct inhibitor of the geranylgeranyl transferase (GGT) enzyme responsible for geranylation of Rho-family proteins. When cells were treated with GGT inhibitor (GGTI), RhoA and Cdc42 pI shifts similar to those resulting from simvastatin treatment were observed (Figure 5). These results provide further evidence that acidic shifts in the isoelectric points of RhoA and Cdc42 after simvastatin treatments were sensitive measures of the isoprenylation status of these proteins.

Isoprenylation of RhoA is inhibited in the brains of simvastatin treated rats and mice

In vitro, we found that simvastatin inhibited the isoprenylation of RhoA and Cdc42 at a concentration of 50 nM (Figure 3). It has been demonstrated that peak levels of simvastatin and its metabolites in the plasma of humans (Prueksaritanont et al., 2001) and the brains of mice (Johnson-Anuna et al., 2005) treated with statins can exceed 200 nM. However, it is unclear whether statins maintain sufficient concentrations *in vivo* to alter protein isoprenylation in the brain. To test whether inhibition of isoprenylation occurs in the brain, we analyzed brains from rats that had been treated with simvastatin or rosuvastatin daily for 30 days. Following isolation of protein lysates from frozen whole brains, we examined the gel migration of RhoA by 2D SDS-PAGE and Western blotting. Whole brain rat RhoA behaved similarly to N2a cell RhoA. In rat brain, RhoA pI was measurably shifted to the acidic (non-prenylated) form in simvastatin-treated brains (Figure 6B), but not in untreated control brains (Figure 6A). Similar changes were observed in rats treated with rosuvastatin (Figure 6C). In untreated animals $91.03 \pm 5.83\%$ of RhoA was isoprenylated, while in rats that received simvastatin and rosuvastatin RhoA isoprenylation decreased to $55.4 \pm 8.17\%$ and $45.5 \pm 5.15\%$, respectively (Figure 6D). These data represent the first reported *in vivo* evidence that simvastatin inhibits isoprenylation in the brain.

To further assess changes in protein isoprenylation after simvastatin administration *in vivo*, simvastatin was given to mice and brain homogenates were analyzed for changes in pI of Cdc42, RhoA, and H-Ras. We found that all three proteins in mice treated with simvastatin exhibited significant acidic shifts, corresponding with the non-prenylated forms of the proteins and supporting the N2a cell-lysate results. Mouse brain RhoA, Cdc42, and H-Ras appeared as multiple grouped bands that spanned a pI range (Figure 7), which is not unusual for 2D gels of small GTPases (Maltese and Sheridan, 1990; Gromov et al., 1996), and corresponded to our findings in cell lines and rats. Utilizing the pI grid for linear pI strips that we create on the PDQuest™ software, the isoelectric points of bands of RhoA, Cdc42, and H-Ras on each respective blot were measured at the acidic and basic ends. We averaged all of the acidic values and also all of the basic values of a group to generate an average range. The pI range for Cdc42 is 5.4–6.4 for the control group versus 4.8–5.8 for the treatment group (Figure 7A); for RhoA the pI is 6.6–7.8 for the control group versus 5.6–6.8 for the treatment group (Figure 7B); and for H-Ras the pI range is 6.7–7.7 for the control group versus 4.9–5.8 for the treatment group (Figure 7C). ANOVA analysis revealed significant differences between control animals (n = 7) and treated animals (n = 8) for Cdc42 (p<0.05), RhoA (p<0.01), and H-Ras (p<0.001).

Simvastatin can also inhibit the farnesylation of numerous proteins including the Ras superfamily GTPases, RhoB, and laminin. We utilized a farnesyl specific antibody to investigate the effects of simvastatin treatment on global farnesylation levels in the mouse brain. Treatment with simvastatin results in the disappearance of anti-farnesylated specific 2D gel protein bands including bands in the molecular weight of small Ras superfamily GTPase (20–30kD) (Figure 8). These data further support the effect of long term simvastatin administration on protein farnesylation in the brains of treated animals.

DISCUSSION

Statins are often thought of as the pinnacle of targeted drug design and are amongst the most widely used drugs in humans. First discovered through screens for HMG-CoA reductase inhibitors (Endo, 1992), statins have since been shown to be highly effective in lowering cholesterol as well as preventing primary and secondary coronary artery disease (Pearson, 1998). However, statins also have been shown to be protective in a number of other conditions including stroke, multiple sclerosis, autism, and AD, even though these diseases are not clearly linked to cholesterol. Therefore, it is becoming increasingly clear that statins may mediate some of their therapeutic benefit independently of their effects on cholesterol, perhaps by modulating other pathways, such as the inhibition of protein isoprenylation, in order to act on inflammatory signaling pathways, protein processing, and secretion pathways.

While the isoprenoid-dependent effects of statins have been well studied *in vitro*, the *in vivo* relevance of these effects has not been as well explored. One hurdle in addressing these questions is that current techniques to assess protein isoprenylation are indirect and cumbersome. GTPases require protein isoprenylation for proper membrane localization, and this has been used as a surrogate marker for GTPase isoprenylation. However, only a few published reports have demonstrated that statins block membrane localization of Rho and

Rac GTPases *in vivo* (Bulhak et al., 2007; Rashid et al., 2009), and no studies have reported statin effects on membrane localization in the brain. The question of the specific effect of statins in the brain is important, as it has been suggested that they have isoprenoid-dependent effects in CNS disorders such as multiple sclerosis, stroke, and AD.

Based on the results of this study, we describe 2D SDS-PAGE techniques as a simple method to monitor protein isoprenylation status. We demonstrate that changes in isoelectric mobility induced by statins are sensitive measures of protein isoprenylation, and that 2D gel techniques can be broadly used to assess protein isoprenylation of Rho- and Rab-family proteins with good sensitivity. For example, we are able to resolve the level of responses depending on simvastatin dose (Figure 3). Furthermore, we are able to distinguish and quantify differing shifts for different protein as shown throughout our studies for RhoA, Cdc42, H-Ras, and Rab proteins. Therefore, our approach can be used to examine differential responses by different proteins, and, with refinement for specific proteins of interest, would be highly sensitive in resolving differential responses.

We found that in the non-treated condition, RhoA and Cdc42 migrated predominantly in a more basic band by 2D SDS-PAGE, representing the carboxymethylated and therefore isoprenylated protein. However, high doses of statins resulted in an almost complete shift of RhoA to the more basic band, representing the non-carboxymethylated and therefore non-prenylated form of the protein. Examinations of Rab protein by 2D SDS-PAGE clearly showed that carboxymethylation is the ultimate mediator of isoelectric mobility shift. Both CXC and CC Rab proteins are modified by isoprenylation and statins inhibit isoprenylation and membrane localization of both classes of proteins (Dai et al., 2007). However, 2D SDS-PAGE only reflects the effects of statins on CXC proteins, which are carboxymethylated, but not on CC proteins, which are not carboxymethylated.

Although 2D SDS-PAGE directly monitors protein carboxymethylation, it still is a sensitive surrogate marker for protein isoprenylation of Rho-family proteins. Protein isoprenylation and carboxymethylation are known to be intimately linked, and statins have been shown previously to inhibit protein carboxymethylation (Haklai and Kloog, 1991; Lerner et al., 1992). To further demonstrate that isoelectric shifting of RhoA and Cdc42 are sensitive measures of changes in protein isoprenylation, we determined that isoelectric mobility changes induced by statins were reversible by supplementation with mevalonate and GGPP, but not FPP. This result correlates with previous evidence that statin inhibition of the membrane localization of GTPases, which is dependent upon isoprenylation, also can be reversed by depleting cellular mevalonate and GGPP. We also further confirmed the link to isoprenylation by examining pI shifts produced by treatment with a GGTI, which we found to be identical to those observed for simvastatin treatment. Overall, our data indicate that isoelectric mobility measured before and after statin treatment reflects the effects of statins on protein isoprenylation.

One of the more significant findings of our studies is that 2D SDS-PAGE may be used to quantify the dose-dependent effects of statins on protein isoprenylation, a topic that has never been addressed previously. Although some studies have shown that statins can inhibit protein isoprenylation at more clinically relevant doses, most studies that examine

isoprenoid-dependent effects of statins have utilized supraphysiologic doses of statins in the 5–10 μ M range (Schulz et al., 2004; Pooler et al., 2006; Kim et al., 2009), and no studies have rigorously addressed dose-dependent effects of statins on protein isoprenylation.

Surprisingly, we found that RhoA and Cdc42 showed almost maximal inhibition of isoprenylation at 500 nM simvastatin, and that statins inhibited isoprenylation of RhoA and Cdc42 at doses as low as 50–200 nM *in vitro*. In young healthy human subjects that received 80 mg simvastatin for one week, simvastatin reached peak plasma concentrations of approximately 200 nM, (Prueksaritanont et al., 2001). This suggests that RhoA may be a clinically relevant target of statin action, as statins likely reach concentrations *in vivo* sufficient to inhibit RhoA isoprenylation. It has been demonstrated that proteins may have differential sensitivity to isoprenoid depletion and prenylation inhibitors (Ostrowski et al., 2007); our results indicate that the 2D SDS-PAGE approach may be a valuable tool for determining dose-response curves for statin effects on the isoprenylation of various GTPases, and thus allow for the detection of the most sensitive targets of statin action.

Of note, as detected by 2D-SDSPAGE, both the prenylated and unprenylated forms of RhoA, and to a lesser extent, Cdc42 migrated in the second dimension in as multiple bands of differing molecular weights. Examination of previous reports of 2D gel electrophoresis of RhoA and Cdc42 does not show multiple molecular weight bands (Backlund, Jr., 1997; Cicha et al., 2004). In addition, multiple molecular weight bands are not seen in 1D SDSPAGE of these proteins (Figure 3A and B). It is possible that increased focusing of proteins by 2D electrophoresis allows for separation of RhoA and Cdc42 that has been phosphorylated or otherwise modified. However, the phenomenon is somewhat variable and not present in all samples (Figure 3B), and most likely represents protein oxidation or other artifact from the prolonged isoelectric focusing step of 2D-SDSPAGE.

We have evaluated statin effects in the brain *in vivo* and found that in rats and mice treated with simvastatin at 10 mg/kg, that the inhibition of RhoA, H-Ras, or Cdc42 isoprenylation can be detected by statin induced 2D SDS PAGE gel shifts. In addition we show that global protein farnesylation is reduced in brains of mice treated with simvastatin. These studies represent the first evidence that statins can inhibit protein isoprenylation in the central nervous system at a dose of 10 mg/kg in rodents. Although pharmacokinetic studies of simvastatin in rats and mice are limited, simvastatin and its active metabolites reach peak plasma levels in rats of either 340 nM or 167 nM after a single oral dose of either 50 mg/kg (Smith et al., 1991) or 25mg/kg (Germershausen et al., 1989), respectively. In mice simvastatin plasma levels are 100 nM one hour after a 10 mg/kg bolus of simvastatin (Higgins et al., 2014). In humans peak concentrations of active statin metabolites can exceed 200 nM after administration of a 40–80 mg dose of simvastatin (Prueksaritanont et al., 2001; Bergman et al., 2004). Thus, the doses used in our study (10 mg/kg) likely represent a clinically relevant dose.

In summary, we have expanded the application of 2D SDS-PAGE to measure protein isoprenylation. We demonstrate this as a simple method for accurate quantitation and evaluation of statin effects on the isoprenylation of multiple proteins, independently and at various doses. Most importantly, we used this approach to detect alterations in protein

isoprenylation in the central nervous system of rodents following the administration of clinically relevant doses of simvastatin.

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Abbreviations used

2D	two-dimensional
AD	Alzheimer's disease
APP	amyloid precursor protein
FPP	farnesyl pyrophosphate
GGPP	geranylgeranyl pyrophosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
Icmt	isoprenylcysteine-O-c transferase
pI	isoelectric point
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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- A direct approach that can measure simvastatin effects on prenylation is explored in vitro and in vivo.
- 2D gel electrophoretic shifts reflected simvastatin inhibition of prenylation of CaaX-motif containing GTPases.
- Inhibition by simvastatin occurred at concentrations as low as 50 nM.
- Simvastatin inhibited RhoA, Cdc42, and H-Ras prenylation in rodent brains following oral administration of clinical doses.

Figure 1. High dose simvastatin treatment leads to change in isoelectric point (pI) of RhoA and Cdc42

N2a neuroblastoma cells were incubated in the presence of vehicle or simvastatin (10 μ M) for 24h. Cells were then harvested and cellular lysates were processed for 2D gel electrophoresis, followed by Western blotting using antibody specific for (A) RhoA or (B) Cdc42. Left and right panels demonstrate 2D and 1D gel blots, respectively. Protein levels and isoelectric point shift of RhoA and Cdc42 bands were assessed by Western blots (probed with anti-GAPDH as a loading control). Blots are representative of 4 separate experiments with 1–3 blots per condition per experiment. Averages given + SD, and ** ($p < 0.01$) connote statistical difference from vehicle control by paired t-test.

Figure 2. High dose simvastatin treatment leads to change in isoelectric point of CXC Rab proteins but not CC Rab proteins

N2a neuroblastoma cells were incubated in the presence of vehicle or Simvastatin (SIM) (10 μ M) for 24h. Cells were then harvested (3 separate experiments) and cellular lysates were processed for 2D gel electrophoresis, followed by Western blotting (1–3 blots per condition per experiment) using antibodies specific for (A) Rab6, (B) Rab4, or (C) Rab11. Blots shown are representative, and ratios of basic to acidic bands are given as averages + SD. ** ($p < 0.01$) connotes statistical difference while NS connotes no significant difference from vehicle treated cells by paired t-test.

Figure 3. Simvastatin treatment leads to changes in isoelectric point of RhoA and Cdc42 in a dose-dependent manner to doses as low as 50 nM

N2a cells were treated with vehicle or with varying concentrations (50 nM–500 nM) of simvastatin (SIM) for 48h. Cells were then harvested and cellular lysates were processed either for 2D or 1D gel electrophoresis, followed by Western blotting using antibody specific for (A) RhoA or (B) Cdc42, and GAPDH as a loading control. Data representative of 3 experiments and 3–4 blots per experiment are shown and quantitations are presented as averages + SD. * ($p<0.05$) and ** ($p<0.01$) connote statistical difference by paired t-test.

Figure 4. Statin induced changes in isoelectric point are reversed by mevalonate and GGPP, but not FPP

N2a cells were incubated for 24h in the presence of (A) vehicle, (B) 200 nM simvastatin (SIM), (C) simvastatin + 2.5 mM mevalonate (MEV), (D) simvastatin + 10 μ g/ml GGPP, (E) simvastatin + 10 μ g/ml FPP, or (F) 200 nM cismethynil. Cells were then harvested and cellular lysates processed for 2D gel electrophoresis, followed by Western blotting using antibody specific for RhoA. Blots shown are representative of 1–2 Western blots from 3–5 experiments and data is given as average + SD. ** ($p < 0.01$) connote statistical difference while NS connotes no difference from vehicle treated cells, by paired t-test.

Figure 5. GGTI treatment results in Rho A and Cdc42 2D gel shifts similar to those observed after simvastatin treatment

N2a cells were treated with vehicle or 10 μ M GGTI-298, a specific inhibitor of the geranylgeranyl transferase I enzyme, for 24 hours. Cells were then harvested and cellular lysates were processed for 2D gel electrophoresis, followed by Western blotting using antibody specific for RhoA or Cdc42. Blots representative of 3 experiments are shown and quantitations are given as averages + SD. ** ($p < 0.01$) connotes statistical difference from vehicle treated cells.

Figure 6. Treatment of rats with simvastatin results in changes of the isoelectric of Rho A in the brain

Adult rats were treated with either (A) vehicle (n=4), (B) treated with simvastatin (SIM), or (C) rosuvastatin (ROS) daily for 30 days at a dose of 10 mg/kg (n=3 for SIM and ROS). After the rats were sacrificed, brains were harvested and protein was isolated. Lysates were processed by standard protocols for 2D gel electrophoresis followed by Western blotting using antibody specific for RhoA. Representative blots of vehicle, simvastatin, and rosuvastatin treated animals are shown. Fractions of isoprenylated RhoA (D) are presented as averages + SD. ** (p<0.01) connotes significant difference from vehicle treated rats, by ANOVA.

Figure 7. Treatment of mice with simvastatin results in shifts in the isoelectric point of Cdc42 and RhoA in the brain

Mice were fed either control chow (top bands) or simvastatin-containing chow (bottom bands) for 30 days (10 mg/kg/day). Mice were sacrificed, brains were harvested, and protein was isolated. Lysates were processed by standard protocols for 2D gel electrophoresis followed by western blotting using antibodies specific for Cdc42, RhoA, or H-Ras.

Isoelectric points (pI) were measured at the (A) acidic (red) and (B) basic (green) ends of the bands of interest. For statistical analysis, the acidic isoelectric point (pI) measurement of the control group was compared to the acidic (A) pI of the treatment group, and the (B) basic pI for untreated with the basic pI for treated, for Cdc42, RhoA, and H-Ras. ANOVA results for basic and acidic shifts are: for Cdc42, $p < 0.05$; for RhoA, $p < 0.01$; and for H-Ras, $p < 0.001$ ($n = 10$ control and 8 treated animals).

Figure 8. Statins cause a decrease of farnesylation, including at the molecular weight and pI that correspond with Ras superfamily GTPases

Representative control (top) and treated (bottom). Mice were sacrificed, their brains harvested and the protein isolated. Lysates were processed by standard protocols for 2D electrophoresis followed by Western blot with an antifarnesyl antibody. Treatment with statins resulted in an overall decrease of farnesylation. When compared with controls, treated blots show loss of detectible farnesylation in the region that corresponds to the Ras superfamily of GTPases (red box). This occurs at the same molecular weight (20–25 kDa) and pI (pH 4.8–7.8) of Ras superfamily GTPases (n=10 control and 8 treated animals).