Statins Reduce Amyloid-β Production through Inhibition of Protein Isoprenylation*

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Stephen M. Ostrowski†‡, Brandy L. Wilkinson†, Todd E. Golde§, and Gary Landreth†‡

From the †Department of Neurosciences, Case Western Reserve University, Cleveland, Ohio 44106 and the §Department of Neuroscience, Mayo Clinic Jacksonville, Mayo Clinic College of Medicine, Jacksonville, Florida 32224

Epidemiological evidence suggests that long term treatment with hydroxymethylglutaryl-CoA reductase inhibitors, or statins, decreases the risk for developing Alzheimer disease (AD). However, statin-mediated AD protection cannot be fully explained by reduction of cholesterol levels. In addition to their cholesterol lowering effects, statins have pleiotropic actions and act to lower the concentrations of isoprenoid intermediates, such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate. The Rho and Rab family small G-proteins require addition of these isoprenyl moieties at their C termini for normal GTPase function. In neuroblastoma cell lines, treatment with statins inhibits the membrane localization of Rho and Rab proteins at statin doses as low as 200 nM, without affecting cellular cholesterol levels. In addition, we show for the first time that at low, physiologically relevant, doses statins preferentially inhibit the isoprenylation of a subset of GTPases. The amyloid precursor protein (APP) is proteolytically cleaved to generate β-amyloid (Aβ), which is the major component of senile plaques found in AD. We show that inhibition of protein isoprenylation by statins causes the accumulation of APP within the cell through inhibition of Rab family proteins involved in vesicular trafficking. Moreover, inhibition of Rho family protein function reduces levels of APP C-terminal fragments due to enhanced lysosomal dependent degradation. Statin inhibition of protein isoprenylation results in decreased Aβ secretion. In summary, we show that statins selectively inhibit GTPase isoprenylation at clinically relevant doses, leading to reduced Aβ production in an isoprenoid-dependent manner. These studies provide insight into the mechanisms by which statins may reduce AD pathogenesis.

Alzheimer disease (AD)§ is a progressive neurodegenerative disease and the most common cause of dementia in the elderly.

The pathologic hallmarks of AD are extraneuronal senile plaques composed of β-amyloid (Aβ) fibrils and intraneuronal accumulations of hyperphosphorylated Tau (1, 2). Aβ is generated by sequential proteolytic processing of the type I transmembrane protein, amyloid precursor protein (APP), by β- and γ-secretases (3, 4). Nascent APP is trafficked via the common secretory pathway and undergoes post-translational modifications including N- and O-glycosylation. Following delivery to the cell surface, APP is trafficked to late endosomes, and either recycled to the cell surface or degraded within the lysosome. APP is cleaved by the β- or α-secretase to generate either a C99 or C83 C-terminal fragment (CTF), respectively. γ-Secretase cleaves the C99 CTF to form Aβ, whereas cleavage of C83 results in the production of a non-amyloidogenic p3 fragment. β- and γ-secretase complexes are found in multiple cellular compartments including the endoplasmic reticulum, late-Golgi/trans-Golgi network, endosomes, and plasma membrane, although there is significant debate with regard to the magnitude of APP processing within individual subcellular compartments and their quantitative contribution to Aβ production (5–8).

The Rab subfamily proteins are critical for vesicular trafficking and have been shown to be involved in regulating Aβ production (9, 10). In particular, Rab1b mediates the transport of APP from the endoplasmic reticulum to the Golgi, where it undergoes glycosylation. Inhibition of Rab1b function through expression of dominant negative forms of this G-protein resulted in impaired trafficking that was associated with inhibition of APP processing and Aβ production (9, 11). Similarly, Rab6 is involved in intra-Golgi trafficking of APP and inhibition of its function by expression of a dominant negative Rab6 leads to a significant reduction of Aβ generation (10). The Rho subfamily of small G-proteins, such as RhoA, Rac, and Cdc42, whereas first recognized for regulating actin-based cytoskeleton rearrangement (12), have been shown to be important elements in a variety of intracellular signaling pathways, including those involved with Aβ production (13, 14). Statins are widely prescribed drugs for treatment of hypercholesterolemia, and act 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, preventing de novo synthesis of cholesterol (15, 16). Epidemiological studies suggest that treatment

ELISA, enzyme-linked immunosorbent assay; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; PIPES, 1,4-piperazineethanesulfonic acid.
with statins reduces the risk of developing AD (17–19). Most work has focused on the cholesterol lowering effects of statins, and statins can reduce Aβ production in vitro through lowering of cholesterol levels. This effect was postulated to result from the sensitivity of β- and γ-secretases to neuronal membrane cholesterol content (20–24). Statins have been shown to decrease Aβ levels and plaque load in some animal models (25, 26), however, it is unclear if lowering of cholesterol is responsible for the observed effects. Several lines of evidence suggest that lowering of cholesterol may not fully explain the protective effects of statins in AD. Notably, the balance of clinical data does not strongly support elevated serum or brain cholesterol as a risk factor for AD (27, 28), and results from animal studies with regard to the involvement of cholesterol in AD pathology is mixed (29–32).

Statins exhibit pleiotropic effects through reduction of isoprenoid intermediates in the cholesterol biosynthetic pathway (33). The isoprenoids geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) are added to the C termini of the Ras superfamily of small G-proteins, including Rho and Rab. Isoprenoid modification is critical for facilitating GTPase interactions with cytoplasmic regulators, cellular membranes, and effectors (34). Thus, the ability of statins to reduce AD risk may arise from inhibition of protein isoprenylation. Cholesterol-independent actions of the statins have already been demonstrated to be important for the clinical benefits of these drugs on cardiovascular disease (35–37), as well as in animal models of central nervous system diseases with an inflammatory component, including multiple sclerosis and ischemic stroke (38–40).

In cell culture, it has been shown that statin inhibition of GTPase isoprenylation causes these proteins to lose their normal membrane association and function (41). However, the effects of statins on protein isoprenylation have not been well studied in neurons. In addition, reports of statin effects on Rab family proteins have been quite limited, although statins have been shown to modulate protein trafficking through inhibition of Rab protein function (42, 43). As APP is trafficked by Rab-dependent mechanisms and perturbation of Rab function is associated with suppression of APP processing and Aβ generation (9–11), we thought it important to examine the effects of statins on Rab isoprenylation, and whether modulation of Rab function by statins may perturb Aβ production.

The physiological levels of statins in the brain have only recently been determined. Johnson-Anuna et al. (44) reported simvastatin reaches concentrations of 300–500 nM in the brains of mice. Effects of statins at these lower, more clinically relevant, doses are not well documented. We report that, in neuronal cell types, statins inhibit the isoprenylation and membrane association of GTPases of the Rho and Rab family at doses of statins as low as 200 nM. Importantly, we show that while at high doses statins universally impair GTPase function, at low doses statins preferentially impair the isoprenylation and membrane localization of only a subset of GTPases. These GTPases may represent specific, clinically relevant targets of statin action. We also show that statins impact APP metabolism through Rho- and Rho-dependent mechanisms, leading to reduced Aβ production. In summary, we show that statins can selectively inhibit the isoprenylation of GTPases at physiologically relevant doses, and suggest that statins may act by cholesterol-independent mechanisms to lower Aβ production and limit AD pathogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Simvastatin and lovastatin were purchased from Calbiochem (La Jolla, CA) and prepared following the manufacturer’s instructions. The statins were converted to the active form by dissolving them in absolute EtOH, followed by the addition of 1 M NaOH to a final concentration of 100 mM. This solution was stored at −20 °C until use. Immediately before use, the statin solution was neutralized with 1 M HCl, and diluted in vehicle (50% EtOH, 5 mM HEPES, pH 7.2). Geranylgeranyl pyrophosphate triammonium salt and farnesyl pyrophosphate triammonium salt were purchased from Biomol (Plymouth Meeting, PA). Mevalonic acid was purchased from Sigma and reconstituted in 100% ethanol. Clostridium difficile Toxin A was purchased from List Labs (Campbell, CA). Cell-permeable C3 exoenzyme was obtained from Cytoskeleton (Denver, CO). The Amplex Red Cholesterol Assay kit was purchased from Molecular Probes (Eugene, OR). Antibodies to APP (22c11) and the APP C-terminal fragment were purchased from Chemicon (Temecula, CA). Antibodies to Rac and Rab4 were obtained from Upstate (Waltham, MA). Antibodies to fliptin and calnexin were obtained from BD Bioscience. The antibody to GAPDH was obtained from Trevigen (Gaithersburg, MD). Antibodies to ERK2, β-tubulin, RhoA, Cdc42, Rab1b, Rab5b, and Rab6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies 6E10 and 4G8 were purchased from Covance (Cumberland, VA). Peroxidase-conjugated secondary antibodies were purchased from GE Healthcare. Cell culture reagents were purchased from Invitrogen.

**Cell Culture**—Mouse N2a (parental) neuroblastoma cells were obtained from American Type Culture Collection (Manassas, VA). N2a.Swe cells were obtained from Dr. Gopal Thi-nakaran (University of Chicago). APPsw-293 cells were obtained from Dr. Robert Vassar (Northwestern University). N2a and APPsw-293 cells were cultured in 50% Opti-MEM, 50% Dulbecco’s modified Eagle’s medium, 5% fetal bovine serum (Hyclone, Logan, UT), and 1% penicillin/streptomycin.

H4.APPWT and H4.HPLAP neuroglioma cells were maintained in Opti-MEM plus 4% fetal bovine serum, 1% penicillin/streptomycin, and 1% Zeocin. H4.APPWT cells overexpress wild type human APP under an actin promoter (45). H4.HPLAP express endogenous levels of APP, but overexpress the human APP C-terminal fragment fused to human placental alkaline phosphatase. All cells were cultured at 37 °C and 5% CO2.

**Neuron Culture**—Primary cultures of cortical neurons were prepared from embryonic day 15–16 C57BL/6 mouse embryos as described (50). Briefly, embryo cortices were dissected, and meninges were removed. Tissue was digested, mechanically dissociated, and suspended in neurobasal medium (B27 supplement, 100 µg/ml penicillin/streptomycin, 0.5 mM glutamine, and 25 µM glutamate), and plated densely onto poly-D-lysine-coated 6-well plates (1 × 106 cells/well). Neurons were maintained under serum-free conditions in neurobasal medium with B27 supplement prior to drug treatment.
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Drug Treatments—Cells were plated at 5 × 10⁵ cells per well in 6-well plates, and allowed to grow for 1 (for 48 h treatment) or 2 days (for 12–24 h treatment) before drug treatment. H4 cells were plated on poly-L-lysine-coated 6-well plates. Cells were then treated with the indicated compounds for 12–48 h.

Western Blotting—Cells were collected and lysed with radio-immunoprecipitation assay (RIPA) buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 40 mM NaF, 0.2% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, and 1 mM Na₃VO₄). Lysates were sonicated for 2 × 10 s on ice and cleared by centrifugation (16,000 × g, 15 min, 4 °C). Protein concentration was determined by the Bradford method (46). The samples were boiled under reducing conditions then resolved on 9% SDS-PAGE gels or NuPage 4–12% BisTris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes. After blocking in a 5% milk or 5% normal goat serum solution, blots were incubated overnight at 4 °C with the indicated antibodies. Bands were visualized by incubation of blots with anti-mouse, rabbit, or goat horseradish peroxidase-conjugated secondary antibodies (1:1000; 90 min at room temperature) and visualized by enhanced chemiluminescence (Pierce). Protein loading was evaluated by probing with anti-ERK2 (1:3000) or anti-GAPDH (1:5000) antibodies. Images were scanned using Adobe Photoshop and band intensities quantify using Image-Pro Plus software package (Media Cybernetics, Inc., Silver Springs, MD). Band densities were normalized for protein loading by comparison with ERK2 or GAPDH band densities. Mean ± S.E. were calculated. Pairwise comparisons were determined using the Tukey-Kramer post hoc test.

Quantification of Secreted Aβ Peptide Levels by ELISA—Following drug treatments, the culture medium was collected, a protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride was added, and medium was centrifuged at 16,000 × g for 15 min at 4 °C. Media from H4.APPWT cells were diluted 1:5 and assayed by ELISA specific for Aβ₁₋₄₀ from BIOSOURCE/Invitrogen (Carlsbad, CA). H4.APPWT cells do not produce detectable levels of Aβ₁₋₄₂.

Membrane Localization and Western Blotting for GTPases—N2a cells were plated into 6-well plates and 24 h later were treated with simvastatin for 24 or 48 h. Cellular fractionation was carried out as described previously (47). Briefly, following statin treatment the cells were lysed by incubation in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, and 10 mM PIPES, pH 7.3) on ice for 15 min followed by a 10-s sonication. Cells were cleared by centrifugation at 500 × g for 5 min at 4 °C. The resulting supernatant was centrifuged for 1 h at 110,000 × g at 4 °C in a Beckman-Coulter ultracentrifuge (SW50.1 rotor). The resulting supernatant was removed (cytosolic fraction), and the membrane pellet was then resuspended in relaxation buffer (membrane fraction). The protein concentration from each fraction was measured using the BCA protein assay from Pierce. Standard Western blotting procedures were used to separate the fractions and transfer them to polyvinylidene difluoride membranes. Blots were probed with antibodies for the individual GTPases, as well as markers for cytosolic (GAPDH, ERK2) and membrane (flotillin, calnexin) fractions.

FIGURE 1. Statin-dependent alteration of G-protein electrophoretic mobility and abundance. N2a cells were treated for 24 h (A) with simvastatin (10 μM) or lovastatin (10 μM), in the presence or absence of mevalonate (250 μM) or for 6 days (B) with 50–100 nM simvastatin. SDS-PAGE was performed on cell lysates, followed by Western blotting using the indicated antibodies. Images shown are representative of at least three independent experiments.

RESULTS

Selective Effects of Statin Treatment on G-protein Isoprenylation and Abundance—We investigated whether statins act uniformly to inhibit the isoprenylation of members of the Rho and Rab families of G-proteins in N2a neuroblastoma cells. We initially monitored statin-mediated effects by decreased electrophoretic mobility of non-prenylated versus prenylated forms of GTPases as examined by SDS-PAGE. It has been reported previously that Ras and Rab (42, 43, 48), but not the Rho family proteins (49) exhibit altered electrophoretic mobility depending upon protein prenylation status, although we have reported altered isoprenoid-dependent Rac electrophoretic mobility in some cell types (41). In statin-treated N2a cells we observed lowered mobility of Rab family proteins after statin treatment but not of the Rho family proteins Rac, Rho, or Cdc42 (Fig. 1A). The statin-dependent change in electrophoretic mobility of these protein families likely reflects the fact that Rab family proteins have two geranylgeranyl moieties added to them, whereas Rho family proteins possess only one lipid moiety. We find that after exposure of N2a cells to high doses of statins (10 μM), the Rab GTPases Rab1b, Rab4, Rab5, and Rab6 are converted entirely into the lower mobility, non-prenylated species (Fig. 1A). Statins reduced the levels of Rab6, requiring overex-
Exposure of the blots to visualize the unprenylated Rab6 band. Similar results were observed in H4 neuroglioma cells (data not shown). Provision of mevalonate allows restoration of the isoprenyl intermediate pools, without significant effects on cholesterol synthesis (20, 21, 25, 50, 51). Consistent with previous reports that show electrophoretic shifts are a result of loss of protein isoprenylation, we observe that changes in electrophoretic protein mobility caused by statin treatment were reversed upon provision of exogenous mevalonate, demonstrating that these effects are dependent upon protein isoprenylation (Fig. 1A). Statins do not alter cellular cholesterol content when serum is present in the media, as has been reported previously (data not shown) (52, 53).

One remarkable outcome of these experiments was the effect of the statins on Cdc42. We find that in N2a cells, statins cause a dramatic increase in Cdc42 levels (Fig. 1A, B). Similar results were found in H4 cells (data not shown). Elevated Cdc42 levels were seen in N2a cells at doses of statins as low as 50 nM (Fig. 1B). Cdc42 mRNA levels were not increased after statin treatment (data not shown), suggesting that isoprenylation may be required for the normal turnover of this protein.

**Statins Block the Membrane Association of Ras Superfamily GTPases**—The isoprenoid modification of the small GTPases is essential for their membrane localization. The membrane association of G-proteins provides a sensitive measure of their prenylation status. Disruption of membrane association of GTPases likely results in loss of protein function because of their inability to associate with membrane-bound effectors. We surveyed the effects of statin inhibition of isoprenoid synthesis on membrane association of newly synthesized Rho and Rab family G-proteins. N2a cells were treated with 10 μM simvastatin or lovastatin for 24 h, in the presence or absence of mevalonate (Fig. 2A). Cells were subjected to biochemical fractionation to isolate membrane and cytosolic fractions. At this statin concentration both simvastatin and lovastatin robustly inhibited the membrane association of all GTPases tested, including Rho family members Rac, RhoA, and Cdc42, and Rab family members, Rab1b, Rab4, Rab5b, and Rab6 (Fig. 2A). Provision of exogenous mevalonate restored membrane localization of all GTPases tested, establishing the reliance of GTPase membrane association on isoprenoids (Fig. 2A). Treatment of N2a cells with 500 nM simvastatin robustly inhibited the membrane association of all GTPases tested (Fig. 2B).

Remarkably, until the recent report by Johnson-Anuna and colleagues (44), the concentrations of statins within the brain were unknown. Simvastatin treatment of mice results in brain levels of 300–500 nM (44). However, the analysis of the effects of statins on protein isoprenylation in vitro has typically employed much higher dosages and little is known about drug effects at physiologically significant concentrations. Protein isoprenylation is directly related to cellular isoprenoid pool size, and at low isoprenoid pool sizes, isoprenoids are primarily incorporated into a subset of GTPases (54). This suggests that statins may differentially alter protein isoprenylation depending on statin dose, because of dose-dependent effects on cellular isoprenoid pool size. We tested whether statins might selec-

![Image](https://example.com/image.png)
tively inactivate GTPases within the physiological dose range.

Strikingly, if physiologically appropriate levels of simvastatin were used to treat the cells we observed a selective effect on the membrane association of the G-proteins. Rab4 and Rab5b membrane localization was not significantly changed by 200 nM simvastatin treatment. Rac and Rab1b localization were decreased after 200 nM simvastatin treatment (Fig. 2C).

**Statins and Toxin A Increase Synthesis of APP in N2a.Swe Cells, but Not in Other Cell Types**—Statins have well documented cholesterol-dependent effects on APP processing and Aβ generation (20–24), however, the isoprenoid-dependent effects of statins on APP processing have not been well studied (52, 55). We investigated the effect of statin inhibition of isoprenylation on APP metabolism and Aβ production.

We investigated whether statins affect the synthesis of APP, because of a previous report that statins act to stimulate APP expression (55). We found that in murine N2a.WT cells, the synthesis of endogenous APP was not significantly increased after simvastatin treatment (Fig. 3A). These studies were extended to examine if statins have different effects on murine versus human APP synthesis. In human H4 neuroglioma cells that express either endogenous hAPP (H4.HPLAP) or hAPP overexpressed under an actin promoter (H4.APPWT), there was no change in APP synthesis after statin or Toxin A treatment (Fig. 3A). Similarly, in APPsw-293 cells, which overexpress hAPP under a cytomegalovirus promoter, we detected no

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**FIGURE 3.** Statins and Toxin A increase APP expression in N2a.Swe cells, but not in other cell types. A–C, pulse-chase experiments. A, N2a.WT, APPsw-293, H4.HPLAP, and H4.APPWT cells were treated for 18 or 24 h with simvastatin (10 μM) or 12 or 18 h with Toxin A (500 ng/ml). B, N2a.Swe cells were treated with simvastatin (10 μM) for 24 h or Toxin A (500 ng/ml) for 12 h. C, N2a.Swe or N2a.WT cells were treated with simvastatin at 300 or 500 nm for 48 h. After treatment, cells were changed to cysteine/methionine-free media for 5 min, followed by media containing 35S-labeled cysteine/methionine for 15 min. APP was immunoprecipitated from lysates. Images shown are representative of at least three independent experiments. For N2a.Swe experiments (B) the APP bands were excised from the gels and incorporated radioactivity was quantified by scintillation counting (n = 6, **, p < 0.01 compared with non-treated sample).

D–F, Western blots and ELISA. D, primary embryonic cortical neurons were treated for 24 h in the presence or absence of simvastatin (10 μM). E, N2a.Swe cells were treated with simvastatin (10 μM) or Toxin A (500 ng/ml) for 12 h. F, media was collected from N2a.WT, H4.APPWT, and APPsw-293 cells after treatment in the presence or absence of simvastatin (1 μM) for 24 h. G, media was collected from non-treated cells. Western blotting was performed against sAPP and sAPPα. Images shown are representative of at least three independent experiments. Aβ1-40 and Aβ1-42 were measured from media by ELISA and quantified (n = 6, **, p < 0.01 compared with non-treated sample).
changes in APP synthesis after statin treatment, concurring with results previously published by Cole et al. (52) (Fig. 3A). In addition, we saw no significant changes in APP levels after 24 h treatment with simvastatin (10 μM) (Fig. 3D), or Toxin A (data not shown) in primary cortical neurons isolated from wild-type C57BL/6 embryos. Thus, in a variety of cell types statins do not affect APP synthesis.

These findings are in contrast with those of Pedrini et al. (55) who have reported that statins cause increased APP synthesis in N2a.Swe cells, which overexpress human Swedish mutant APP under a cytomegalovirus promoter, a cell model used widely to examine APP processing. We observed similar results, and show that statin treatment of APP in N2a.Swe cells increased the expression of APP, consistent with the previous report (55). Pulse-chase experiments confirmed that this was due to increased APP synthesis (Fig. 3, B and C). In N2a.Swe cells, simvastatin caused increased APP synthesis at doses as low as 300 nM (Fig. 3C). Toxin A, a robust and specific Rho family inhibitor often used to delineate the Rho family-dependent effects of statins (56), also increased APP synthesis in N2a.Swe cells (Fig. 3B), whereas having no affect on APP synthesis in other cell types (Fig. 3, A and B). Interestingly, we demonstrate that increased APP synthesis led to a concomitant increase in sAPP and Aβ1–40 and Aβ1–42 production (Fig. 3E). Thus, in N2a.Swe cells we found that the predominant effect of statin treatment was to increase APP expression, sAPP and Aβ secretion. These data indicate that inhibition of Rho family G-protein function resulted in a stimulation of APP expression in N2a.Swe cells, but not in other cell types.

Similar to observations by Pedrini et al. (55), we observed that statins preferentially increased the secretion of sAPPα over total sAPP in N2a.Swe (Fig. 3F). However, in H4.APPWT and APPSw-293 cells we observed no increases in either sAPP or sAPPα after statin treatment (Fig. 3F). In addition, we found that whereas N2a.Swe produced roughly comparable levels of sAPP to H4.APPWT and APPSw-293 cells, that N2a.Swe cells produced extremely low amounts of sAPPα (Fig. 3G). We conclude that in N2a.Swe cells the increased expression of the hAPP transgene and specific up-regulation of sAPPα by statins is unique to this specific cell line and is not representative of the response of other cell lines and primary neurons, to statin treatment.

**Statin Inhibition of Rho and Rab Decreases Aβ**

FIGURE 4. Statin, but not Toxin A, treatment leads to accumulation of APP in N2a cells, perturbing APP processing and decreasing Aβ secretion. N2aWT cells (A and B) were treated for 24 h with simvastatin (10 μM) or lovastatin (10 μM) in the presence or absence mevalonate (250 μM) or 24 h (C) with simvastatin (10 μM) in the presence or absence of GGPP (1 μg/ml) or FPP (1 μg/ml) for 48 h (D) with simvastatin at doses of 300 or 500 nM. APP, CTF, and GAPDH were measured from lysates by Western blotting. sAPP levels were measured from the media by Western blotting. Images shown are representative of at least three independent experiments. Aβ1–40 levels were measured in the medium by ELISA and quantified (n = 3–4, *, p < 0.05; **, p < 0.01 compared with non-treated sample).

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Statins did not change the levels of sAPP generated by these cells. Interestingly, the accumulation of APP and the decrease of Aβ secretion was observed at statin doses as low as 300 nM (Fig. 4D). Overall, these data demonstrate that statin treatment led to the accumulation of APP through inhibition of protein geranylgeranylation, but independent of Rho family inhibition. We conclude that as APP is trafficked within the cell through Rab-dependent mechanisms, it is likely that inhibition of Rab isoprenylation by statins alters APP trafficking leading to APP accumulation.

**Inhibition of Rho by Statins or Toxin A Decreases CTF Levels and Reduces Aβ Secretion**—In primary neurons, treatment with simvastatin or lovastatin for 24 h resulted in no detectable changes in APP levels (Fig. 3D). We conclude that there are likely cell type-specific effects of statin actions, and thought it relevant to look at statin actions in other cell types. We examined APP processing in two human neuroglioma H4 cell lines: 1) H4.APPWT, which overexpresses wild type human APP under an actin promoter, and 2) H4.HPLAP, which express endogenous human APP, but overexpresses the human APP C-terminal C100 fragment. We show that whereas H4.APPWT cells expressed 5–10-fold more APP than H4.HPLAP, both cell lines expressed similar levels of CTFs, suggesting that in H4.HPLAP cells that α- and β-cleavage of full-length APP does not make a quantitatively significant contribution to CTF generation (Fig. 6A).

We examined the effects of statins on APP accumulation in H4 cells. Interestingly, simvastatin and lovastatin treatment caused a slight increase in APP accumulation in these cells (Fig. 6B). Pulse-chase analysis demonstrated that simvastatin, but not Toxin A, caused the accumulation of newly synthesized APP, excluding the role of Rho family proteins in APP accumulation (Fig. 5B). This accumulation of APP by statins was rescued by mevalonate and GGPP, but not FPP, indicating that the statin-induced APP accumulation was dependent on inhibition of protein geranylgeranylation (Fig. 6, B and D). Similar to effects seen in N2a cells, statin treatment in H4 cells led to accumulation of APP, likely through Rab-dependent mechanisms, but these effects were much smaller in magnitude than those observed in N2a cells.

Surprisingly, statin treatment led to reduced levels of APP-CTFs in H4 cells. The non-amyloidogenic C83 fragment is generated from α-secretase cleavage of APP, whereas the amyloidogenic C99 fragment is generated from β-secretase cleavage. Reduction of CTF levels may thus represent a mechanism of reducing Aβ production. Statin-mediated reduction of CTF levels was rescued by mevalonate and GGPP, but not FPP, and was thus dependent upon inhibition of geranylgeranylated proteins (Fig. 6, B–D). Toxin A treatment also caused a decrease of both α- and β-CTF levels (Fig. 7A). Toxin A inhibits Rho family proteins, although not affecting Rab function, indicating that decreases in CTF levels after statin treatment were due to inhibition of Rho protein function. Toxin A treatment also decreased CTF levels in N2a.WT and APPsw-293 cells (data not shown). In addition, CTF levels were reduced by treatment of cells with C3 exoenzyme, an inhibitor specific to RhoA, -B, and -C (Fig. 7C).
Interestingly, whereas Toxin A reduced both \( H9251 \) - and \( H9252 \) -CTFs, statin treatment at higher doses (10 \( \mu \)M) decreased \( H9251 \) - but not \( H9252 \) -CTFs. Statin or Toxin A treatment led to a statistically significant reduction of \( H9251 \) -CTF levels by 50% (Figs. 6C and 7A). We hypothesized that at higher statin doses, Rab-dependent effects on APP and CTF accumulation masked the Rho-dependent effect of statins on reduction of \( H9252 \) -CTFs. Treatment with lower doses of statins (0.5–1 \( \mu \)M) for longer times (5 days) decreased both \( H9251 \) - and \( H9252 \) -CTFs while having no effects on APP accumulation (Fig. 6F). These data suggest that at high doses, Rab-dependent effects on APP and CTF accumulation, as seen in N2a cells, overlap with Rho-dependent effects on decreased CTF levels, whereas at lower statin doses Rho-dependent effects predominate.

Importantly, statin and Toxin A-mediated reduction of CTF levels were observed in both H4.APPWT and H4.HPLAP cells, suggesting that the ability of these agents to decrease CTF levels occurred at steps following APP cleavage that resulted in the formation of CTFs (H4.APPWT) or expression from the C100 transgene (H4.HPLAP) (Figs. 6, B–E, and 7A). In addition, secretion of total sAPP and sAPP\( \alpha \) did not change in H4 cells after simvastatin or Toxin A treatment (Figs. 6C and 7A). These data support our conclusion that inhibition of Rho by statins or Toxin A did not affect the generation of CTFs via APP cleavage, but instead increased their subsequent metabolism.

We examined effects of statins and Toxin A on \( \beta \) secretion in H4 cells. Both statin and Toxin A treatment significantly reduced \( \beta \) secretion by about 30% (Figs. 6G and 7B). Overall, our data demonstrates that inhibition of Rho family members RhoA, -B, and/or -C by statins, Toxin A, and C3 exoenzyme resulted in decreased CTF levels, leading to decreased production of \( \beta \).

**Statin Inhibition of Rho and Rab Decreases \( \beta \)**

We observed that statin and Toxin A-mediated G-protein inactivation leads to decreased CTF levels in H4 cells and that this is likely due to the metabolism of CTFs after they have been generated. We hypothesized that after the CTFs are produced, statin or Toxin A treatment stimulated the proteolytic degradation of CTFs. CTFs have been shown to be degraded within the lysosome (57–59) as well as by...
the proteasome (60, 61). To our knowledge, signaling pathways that may regulate CTF degradation pathways have not been described.

Treatment of H4.HPLAP cells with the proteasomal inhibitor MG132 led to significant increases in CTF levels with little change in APP levels, demonstrating that CTFs are degraded by the proteasome (Fig. 8A). Chloroquine is a weak base that impairs lysosomal function by increasing lysosomal pH (62). Treatment of H4.HPLAP cells with the lysosomal inhibitor chloroquine increased CTF levels with little change in APP levels, demonstrating that CTFs are also degraded by the lysosome (Fig. 8A). We treated H4 cells with Toxin A or statins in the presence or absence of MG132 or chloroquine. MG132 treatment did not impair Toxin A-mediated CTF degradation (Fig. 8, A and B). Chloroquine blocked both Toxin A and statin-mediated reductions of CTF levels (Fig. 8, A and B). We conclude that statins and Toxin A, through inhibition of Rho family proteins, increased degradation of CTFs through a lysosomal dependent mechanism.

**DISCUSSION**

The primary focus of this study was to determine the mechanistic basis of the salutary effects of statins on AD risk. We focused on the pleiotropic actions of these drugs because there is little compelling evidence that statin action in AD is a result of their cholesterol lowering actions. Moreover, relatively little is known about how isoprenoids might participate in disease pathogenesis. One important confound in the investigation of statin action in the brain has been that the concentration of statins that are achieved in the brain after oral administration was unknown. It was only recently reported that the physiologically relevant statin levels in the brain are in the 300–500 nm range, and thus much of the literature concerning the biological effects of these drugs may not reflect their actions in vivo. This study has two principal conclusions. First, we demonstrate that at physiologically relevant concentrations statins selectively inactivate only a subset of G-proteins. Second, we find that the inhibition of Rab family isoprenylation results in inhibition of APP trafficking and Aβ production, whereas inhibition of members of the Rho family suppresses Aβ production through catabolism of APP CTFs through the lysosome.

This study provides a detailed analysis of the effects of statin-mediated inhibition of GTPase isoprenylation on APP processing. This represents a departure from most previous investigations, which have examined the role of cholesterol depletion by statins on Aβ production. To focus on isoprenylation-dependent effects of statins, we utilized well described properties of the isoprenoid and cholesterol pathways. In cell culture, statins inhibit de novo cholesterol synthesis, but cellular cholesterol levels are maintained from lipoprotein uptake from serum in media (52, 53). Moreover, we have verified that the statin-mediated changes were due to reduction in protein isoprenylation by demonstrating complete reversal of statin effects upon provision of exogenous mevalonate at concentrations that do not affect cholesterol synthesis (20, 21, 25, 50, 51) or through provision of GGPP.

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**FIGURE 7.** Toxin A treatment reduces CTF levels and Aβ production in H4 cells. A, H4.APPWT or H4.HPLAP cells were treated in the presence or absence of Toxin A (500 ng/ml) for 12 h. APP, GAPDH, and CTF levels were measured from cellular lysates by Western blotting. sAPP and sAPPα were evaluated from cell culture media by Western blotting. Images shown are representative of at least three independent experiments. Graph represents quantification of CTFα levels as measured by Western blotting (n = 8, *, p < 0.05; **, p < 0.01 compared with non-treated sample). B, H4.APPWT cells were treated for 12 h with Toxin A (500 ng/ml). Secreted Aβ1–40 levels were measured from conditioned cell media by ELISA and quantified (n = 8, **, p < 0.01 compared with non-treated sample). C, H4.HPLAP cells were treated with C3 exoenzyme (10 μg/ml) for 24 h. APP, GAPDH, and CTF levels were measured by Western blotting. Images shown are representative of at least three independent experiments.
Proteins that are affected by drug treatment. Moreover, the effects of high dose statin treatment on protein isoprenylation may not be representative of statin effects at physiologically relevant concentrations.

We demonstrate, to our knowledge for the first time, that physiologically relevant concentrations of statins preferentially inhibit the membrane association and isoprenylation of a distinct subset of GTPases. The rationale for our experiments were based on a study reported by Rilling and colleagues (54) who found that the isoprenylation status of the individual G-proteins exhibited isoform-dependent sensitivity to alteration of the size of the intracellular isoprenoids pools. We provide direct evidence for preferential inhibition of isoprenylation of a subset of G-proteins by statins. We demonstrate that after treatment with statins in a physiologically relevant dose range (200 nM), that the membrane associations of a subset of GTPases (e.g. Rac and Rab1b) are robustly inhibited, whereas the membrane associations of other GTPases (e.g. Rab4 and Rab5b) are unchanged (Fig. 2, C and D). We conclude that only a subset of GTPases are biologically relevant targets of statin action.

The basis of the selectivity of statin action is unclear. It has been shown that primary sequence differences in Ras and Rab isoforms cause them to interact uniquely with the transferase enzymes, resulting in different reactivity with the enzyme (65, 66). It is likely that individual GTPases have intrinsic properties that regulate how efficiently they are isoprenylated by the individual transferases. A more detailed analysis will be needed to assess the magnitude of these differences and what role these mechanisms play in regulating the sensitivity of GTPase isoprenylation to statin treatment.

**Statins Increase Synthesis of APP Only in N2a.Swe Cells**—We observed that in most cell lines and primary neurons that statins and Toxin A do not affect the synthesis of APP (Fig. 3A). These observations confirm results published by Cole et al. (52), but conflict with those of Pedrini et al. (55) who found that statins increase APP expression in N2a.Swe cells. The present study resolves this controversy. We demonstrate that statins and Toxin A increased APP synthesis by 2–2.5-fold in N2a.Swe cells through inhibition of Rho family proteins (Fig. 3, B and C). These data indicate that inhibition of Rho proteins up-regulate synthesis from this promoter in N2a.Swe cells. Our data suggests that N2a.Swe cells respond to statin treatment in an atypical manner and this is a caution in the use of these cells to study statin effects on APP processing or sAPPα shedding.

**Statins Accumulate APP through Inhibition of Rab Protein Function**—Whereas Aβ has been shown to be processed in various intracellular compartments, the bulk of evidence suggests that Aβ is produced primarily in the trans–Golgi network and recycling compartments (3, 11, 67). Targeting APP away from the trans–Golgi network with an endoplasmic reticulum retention motif, or by pharmacologically blocking Golgi trafficking,
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decreases Aβ production (9, 11, 68–71), and conversely targeting APP to the trans-Golgi network increases Aβ production (69, 71). Rab proteins are involved in APP trafficking, and in particular Rab1b mediates endoplasmic reticulum-Golgi trafficking (9, 11), suggesting that inhibition of Rab function by statins may modulate APP trafficking and Aβ production.

In N2a.WT cells statin treatment led to the accumulation of newly synthesized APP (Fig. 4) that was accompanied by the accumulation of CTFs and a 30–40% reduction in Aβ (Fig. 4, A and B). We conclude that impairment of APP trafficking in N2a cells causes accumulation of APP in subcellular vesicular compartments, leading to reduced production of Aβ. Importantly, we observed these same effects on APP accumulation and reduction of Aβ at low, physiologically relevant, statin doses (Fig. 4D).

Cole et al. (52) reported that statins caused accumulation of APP in APPsw-293 cells and other cell types. We have extended these observations by demonstrating that statins impair maturation of APP, as assessed by pulse-chase analysis (Fig. 5). We also provide direct evidence that statins impair Rab protein isoprenylation, and provide data strongly suggesting that the cellular accumulation of APP is due to impairment of Rab-dependent mechanisms (Figs. 1 and 2). We observe that statin-mediated accumulation of APP is dependent upon protein geranylgeranylation (Fig. 4C). The observation that Toxin A, which selectively inhibits the Rab family proteins (Rho, Rac, and Cdc42) (72), has no effect on statin-mediated APP accumulation, rules out a significant role for Rab family proteins on APP trafficking (Fig. 5). However, the Rab protein TC10 has been shown to be involved in cystic fibrosis transmembrane conductance regulator (73) and GLUT4 (74) protein trafficking (9, 11), suggesting that inhibition of Rab function by statins affects additional members of this family.

The effects of statins on APP trafficking and Aβ production in N2a cells are strikingly similar to those previously reported after inhibition of Rab1b. Inhibition of Rab1b by dominant negative forms of this G-protein has been shown to impair the maturation of APP, as detected by pulse-chase analysis, resulting in decreased Aβ secretion (9, 11). Interestingly, Rab1b also caused the retention of APP CTFs within the endoplasmic reticulum (26). This suggests that Rab1b inactivation mislocalizes APP intracellularly, leading to reduced Aβ production. Similarly, Rab6 function has been shown to reduce Aβ production, however, no effects were reported on APP trafficking or localization (10). Significantly, we show that Rab1b isoprenylation is inhibited by statins at doses as low as 200 nM (Figs. 1 and 2). As the effects of statin treatment on APP trafficking are similar to those reported for Rab1b inhibition, it is likely that inhibition of Rab1b is at least in part responsible for the observed effects. However, there are over 30 Rab family proteins involved in vesicular trafficking and it is probable that statins affect additional members of this family.

We demonstrate that in statin-treated N2a cells that APP accumulation is accompanied by decreased Aβ secretion. This finding conflicts with the report of Cole et al. (52) that following statin treatment of APPsw-293 cells, secreted Aβ levels are not changed by statins, whereas intracellular Aβ levels are increased. Moreover, these authors report that statins increased β-CTF levels in APPsw-293 cells, but that α-CTF levels were not dramatically changed. We find that in N2a.WT cells, both α- and β-CTF levels are increased by statin treatment. Thus, in different cell types, statin treatment leads to differential processing of accumulated APP. It is important to note that the experiments reported by Cole et al. (52) utilized the Swedish mutant APP, which is preferentially cleaved by β-secretase (75), and thus may be processed differently than wild type APP. Overall, these data suggest that the statin-mediated APP accumulation has cell type and transgene-dependent effects on Aβ production. We argue that cellular accumulation of APP by Rab-dependent mechanisms may represent a mechanism by which statins limit Aβ production.

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