The 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitor pravastatin enhances neurite outgrowth in hippocampal neurons

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Abstract
Epidemiological studies demonstrate a relationship between statin [3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor] usage and reduced risk of developing Alzheimer’s disease. To determine whether statins affect neuronal development, we treated cultured rat hippocampal neurons with pravastatin. After 4-48 h of treatment, pravastatin significantly increased the number of neurites produced by each cell and caused a corresponding increase in levels of the membrane phospholipid phosphatidylcholine. Pravastatin treatment also significantly increased neurite length and branching but did not affect cellular cholesterol levels. Co-incubation with mevalonate, but not cholesterol, abolished the stimulatory effect of pravastatin on neurite outgrowth. Treatment of neurons with isoprenoids also abolished the effect of pravastatin on neurite growth, suggesting that pravastatin may stimulate neuritogenesis by preventing isoprenylation of signaling molecules such as the Rho family of small GTPases. A specific inhibitor of geranylgeranylation, but not farnesylation, mimicked the stimulatory effect of pravastatin on neuritogenesis. Pravastatin treatment significantly decreased levels of membrane-associated RhoA. These data suggest that pravastatin treatment increases neurite outgrowth and may do so via inhibiting the activity of geranylgeranylated proteins such as RhoA.

Keywords: cholesterol, 3-hydroxy-3-methylglutaryl coenzyme A reductase, isoprenylation, neuritogenesis, Rho, statin.

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that results in memory loss and cognitive decline. In addition to their characteristic amyloid-β peptide (Aβ)-enriched amyloid plaques and neurofibrillary tangles, the brains of AD patients display pathologies such as synaptic loss and neuritic dystrophy (McKee et al. 1991). In vitro, the presence of Aβ peptide alone can cause retraction and deterioration of neuritic processes (Tohda et al. 2004). In transgenic mice, it is possible to reverse neuritic dystrophy through infusion of anti-Aβ antibody (Brendza et al. 2005). However, the mechanism by which Aβ may cause neuritic deterioration is not known. Treatments that restore neurite growth in Alzheimer brains might be efficacious in repairing or preventing neurodegeneration in this disease.

Epidemiological studies have found an inverse relationship between usage of the cholesterol-lowering drugs and risk of developing AD (Wolozin et al. 2000; Rockwood et al. 2002; Zamrini et al. 2004; Dufouil et al. 2005; for review see Kivipelto et al. 2005). Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA into mevalonate; this is the rate-limiting step in cholesterol biosynthesis (Hamelin and Turgeon 1998). However, reduction of cholesterol levels may or may not correlate with reduced risk of AD in patients taking statin drugs (Wood et al. 2003; Wolozin 2004; Eckert et al. 2005). Furthermore, statin usage is associated with a decreased risk of depression and anxiety, which is not correlated with plasma cholesterol levels (Young-Xu et al. 2003). Oral administration of statins, in addition to inhibiting cholesterol synthesis, also affects gene expression in the mouse brain (Johnson-Anuna et al. 2005).
Thus, statins might prevent onset of AD by a mechanism independent of their effect on cholesterol.

Mevalonate is required, not only for cholesterol synthesis, but also for formation of the isoprenoids geranylgeranylpseudophosphate (GGPP) and farnesylpyrophosphate (FPP) [reviewed by Rando (1996)]. Members of the Rho family of small GTPases are activated when isoprenylated by these compounds (Zhang and Casey 1996), and regulate cell growth and neurite extension by reorganizing the actin cytoskeleton [reviewed by Takai et al. (2001)]. RhoA is negatively coupled to cell growth; thus, prevention of RhoA isoprenylation increases neurite extension (Sebok et al. 1999). Statins inhibit isoprenylation of Rho proteins in neuronal cells (Meske et al. 2003; Pedrini et al. 2005) and cultured microglia (Bi et al. 2004; Cordle and Landreth 2005). However, it is not known whether statins can affect neurite outgrowth, and whether such an effect might be mediated by regulation of Rho activation.

In the present study, we investigated the effect of the HMG-CoA reductase inhibitor pravastatin on neurite growth in primary cultured hippocampal neurons. We found that treatment of neurons with pravastatin enhances neurite number, length and branching, and that this effect is probably mediated by inhibition of Rho isoprenylation. These data suggest that the ability of statins to reduce AD risk may be as a result, in part, of inhibition of isoprenoid formation and subsequent prevention of neuritic dystrophy and deterioration.

Materials and methods

Preparation of hippocampal cultures

Cultures of rat hippocampal neurons were prepared as described by Malgaroli and Tsien (1992), with a few modifications. Rat pups, ages P1 to P3, were decapitated. The brains were placed in a dish containing Hank's balanced salt solution Ca++ and Mg++ free (HBSS; Sigma, St Louis, MO, USA) with fetal bovine serum (20%). The cells were then plated onto 35-mm cell culture dishes (-150 cells/mm²) containing Hank's balanced salt solution Ca++ and Mg++ free, coated with Matrigel (Collaborative Research Inc., Bedford, MA, USA). After 48 h of incubation, the media were removed and the cell pellet re-suspended in minimum essential medium (Gibco, Rockville, MD, USA) containing glutamine (1 mM), GGPP (20 µM), FPP (20 µM), GGTI-286 (50 µM), mevastatin (1 µM), GGPP (20 µM), FPP (20 µM), GGTI-286 (50 µM), farnesyltransferase inhibitor (FTI)-277 (50 µM), or methyl-β-cyclodextrin (MβCD; 50 µM). Once a compound was added to the culture it was not removed, with the exception of MβCD. To acutely extract cholesterol from the cell membrane, we incubated the neurons for 30 min in media containing 50 µM MβCD. After the incubation period, we removed the MβCD-containing media, replaced it with fresh media, and analyzed neurite growth 24 h later. All treatments longer than 48 h were refreshed every 48 h. All compounds were from Sigma unless indicated otherwise.

Neurite morphology

For experiments examining the effect of a treatment on neurite development, neurons were incubated with media containing the appropriate test compound for 2-48 h. Following the treatment period, phase-contrast digital images of the cells were taken using a Zeiss Axioplan 2 Fluorescent/Phase-Contrast microscope (Carl Zeiss, Germany), as described by Pooler et al. (2005). Briefly, digital images of neurons were analyzed for neurite number, length, and branching. A neurite was defined as a process that is longer than the width of the cell body. These measurements were taken by an assistant blind to experimental treatment. Four to six images were taken per dish; approximately 100 neurons were quantified per treatment group per experiment. Each experiment was performed in triplicate, unless otherwise noted.

Phospholipid quantification

Following experimental treatment, the media were removed, and the cells harvested in methanol and disrupted using a sonicator (Ultrasonic Inc.). An aliquot of homogenate was removed for total protein determination. The remaining homogenate was extracted with a methanol: chloroform: water (1 : 2 : 1) mixture. After centrifugation, the aqueous phase was aspirated and the organic phase dried on a pre-channeled silica plate (Adsorbasil Plus 1, Alltech, Nicholasville, KY, USA). The lipids were separated by developing the plate for 2 h using a chloroform: ethanol: triethylamine: water (30 : 34 : 30 : 8) mobile phase. After separation, the bands were visualized under UV light by spraying the plate with a diphenyl-hexatriene/ether solution. The bands of interest were scraped into glass tubes and subjected to perchloric acid digestion (3 h; 160°C) followed by determination of total phosphate content as described by Li and Wurtman (1998). Phosphate values were normalized for total protein content.

Cholesterol determination

Total cellular cholesterol levels were determined in neurons grown on 35-mm dishes, using either the Amplex Red assay (Molecular Probes, Eugene, OR, USA) or the Infinity reagent (Sigma), as per the manufacturers' instructions. Results were normalized to total protein content per sample.

Cell fractionation and immunoblotting

For whole-cell lysates, neurons were scraped into 100 µL lysis buffer (60 mM Tris-HCl, 20% glycerol, 1 mM diothiothreitol, plus a protease inhibitor cocktail containing: 1 mM AEBSF, 8 µM aprotinin, 500 µM bestatin, 15 µM E64, 200 µM leupeptin, 10 µM pepstatin A) and boiled for 10 min. For subcellular fractionation, cells were processed as described in Tanaka et al. (2000), with a few...
modifications. Briefly, neurons were harvested in hypotonic buffer (5 mM Tris-HCl, 5 mM NaCl, 1 mM CaCl₂, 2 mM EGTA, 1 mM MgCl₂, 2 mM dithiothreitol) including the protease inhibitor cocktail described above. The cell suspension was sonicated using a cell disrupter (Ultrasonic, Plainview, NY, USA) and separated into membrane and cytosolic fractions by centrifugation (100,000 g, 30 min). The pellet containing the membrane fraction was resuspended in 50 μL lysis buffer and boiled for 10 min. For immunoblotting, the total amounts of protein in each sample were determined by the bicinchoninic acid assay (Smith et al. 1985) (Sigma). Equal amounts of protein were loaded and separated using 4–20% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE; Cambrex, Rockland, ME, USA). Proteins were then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked in 5% bovine serum albumin/Tris-buffered saline (0.15% Tween 20) for 2 h at room temperature. The membranes were washed briefly in TBST and incubated overnight (4°C) in rabbit polyclonal RhoA primary antibody or mouse monoclonal β-tubulin primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA). Following this incubation, membranes were washed with TBST (3 x; 10 min) and incubated for 1 h with a peroxidase-linked secondary antibody. Protein–antibody complexes were detected and visualized using a chemiluminescence system (Amersham, Piscataway, NJ, USA) and Kodak X-AR film as described by the manufacturer. Specificity of RhoA staining was confirmed using a blocking peptide: when the primary RhoA antibody was combined and analyzed using the public domain NIH Image program (developed at the US National Institute of Health) and available on the Internet at http://rsb.info.NIH.gov/NIH-IMAGE/. The optical densities of the experimental groups are normalized to the average optical density of the control group. All values are expressed as fold-basal increases or decreases compared with controls.

Data analysis
Data were analyzed using analysis of variance (ANOVA) using treatments as the independent variable. When differences were detected (p ≤ 0.05), the means were separated using the Newman–Keuls test. Values represent mean ± SEM.

Results

The HMG-CoA reductase inhibitor pravastatin increases neurite outgrowth in hippocampal neurons

Pravastatin treatment significantly enhanced neurite outgrowth in rat hippocampal neurons [7 days in vitro (DIV)]. Twenty-four-hour treatment with pravastatin (50–100 μM) significantly increased the number of neurites produced per cell (Figs 1a and c). Pravastatin (100 μM) visibly enhanced neuritogenesis as early as 4 h after the start of treatment (Fig. 1b), relative to neurite outgrowth observed in control cells. Neuritic processes are highly enriched with membrane phospholipids; hence an increase in neurite number might be associated with increased levels of phosphatidylcholine, a major component of the cell membrane. We found that 24-h treatment of neurons with pravastatin (50 μM) significantly increased phosphatidylcholine levels (Fig. 1d).

Pravastatin increases neurite length and branching

Besides quantifying the number of neurites produced per cell, we also measured the effect of pravastatin on neurite length and branching. Following 24-h pravastatin treatment (100 μM), both the number of branches per neurite and neurite length were significantly enhanced (Figs 2a and 3), compared with branching and length observed in untreated cells.

Pravastatin does not alter total neuronal cholesterol levels

Primary cultured rat hippocampal neurons (7 DIV) were incubated for either 4 or 24 h in media containing the HMG-CoA reductase inhibitor pravastatin (100 μM) or pravastatin plus cholesterol (20 μg/mL). As a control, additional dishes of cells were treated for 30 min with MβCD (50 μM), which extracts cholesterol from the cell membrane. Pravastatin treatment did not decrease total cellular cholesterol levels, whereas addition of exogenous cholesterol to the cell media significantly enhanced cholesterol levels after 4 h but not 24 h (Fig. 3). MβCD significantly depleted cholesterol levels compared with levels in untreated cells.

The effect of pravastatin on neuritogenesis is not prevented by cholesterol treatment

Pravastatin, as an HMG-CoA reductase inhibitor, blocks formation of mevalonate, which is required not only for cholesterol synthesis, but also for formation of isoprenoids (Stamatakis et al. 2002). In order to determine whether pravastatin might be stimulating neurite outgrowth by inhibiting cholesterol synthesis, we co-incubated neurons with pravastatin (100 μM) and cholesterol (20 μg/mL). We found that cholesterol did not block the increase in neurite number or length caused by pravastatin (Figs 4a and c); cholesterol alone had no effect on neuritogenesis. Furthermore, depletion of cholesterol by (30-min treatment) did not mimic the effect of pravastatin on neurite outgrowth; instead, it significantly inhibited neurite outgrowth relative to growth from control cells (Fig. 4a). However, co-incubation of neurons with mevalonate (1 mM) prevented stimulation of neurite growth by pravastatin (Figs 4b and c), suggesting that the effect of pravastatin is as a result of specific inhibition of mevalonate synthesis, and not of inhibition of cholesterol synthesis.

Pravastatin enhances neuritogenesis via inhibition of isoprenylation

Mevalonate is required for synthesis of isoprenoid lipids like FPP and GGPP. Rho-GTPases are activated when GGPP attaches to their C-terminus, allowing the GTPase to associate with the cell membrane. In order to determine whether
pravastatin's stimulation of neuritogenesis involves inhibition of isoprenylation, we co-incubated neurons with pravastatin (100 μM) and either FPP (20 μM) or GGPP (20 μM). Both FPP and GGPP blocked the effect of pravastatin on neurite outgrowth (Fig. 5a). Furthermore, treatment of neurons with the geranylgeranyltransferase inhibitor GGTI-286 (50 μM) mimicked the effect of pravastatin and significantly enhanced both neurite number (Fig. 5b) and neurite length (Fig. 5c). FTI-277 (50 μM), a specific farnesyltransferase inhibitor, had no effect on neurite growth (Fig. 5b).

Pravastatin affects intracellular localization of RhoA
RhoA is an active GTPase when bound to the cell membrane, a process that requires isoprenylation by GGPP. Following 4-h pravastatin treatment, cell lysates were collected and centrifuged into membrane and cytosolic fractions. Levels of membrane-bound RhoA were significantly decreased by 4-h pravastatin treatment (Fig. 6a), whereas cytosolic levels of RhoA were significantly increased (Fig. 6b) relative to levels in control cells.

Discussion
These data show that the HMG-CoA reductase inhibitor pravastatin (50–100 μM) enhances neurite outgrowth (Figs 1a, b and c), neurite length (Fig. 2a) and neurite branching (Fig. 2b) in cultured hippocampal neurons. This effect occurs as early as 4 h after exposure and continues for up to 2 days (Fig. 1b). The increase in neurite outgrowth is associated with increased levels of the membrane phospholipid phosphatidylcholine (PC) but not with changes in total levels of cellular cholesterol (Fig. 3). The stimulatory effect of pravastatin on neurite growth is not affected by the additional of exogenous cholesterol (Figs 4a and c), whereas co-treatment with mevalonate (Figs 4b and c) significantly inhibits this effect. These results suggest that pravastatin may stimulate neuritogenesis, not by inhibiting cholesterol synthesis, but rather by a cholesterol-independent mechanism. To determine whether pravastatin might be stimulating neurite outgrowth by inhibiting formation of isoprenoids known to regulate Rho-GTPases, we co-treated neurons with pravastatin and either GGPP or FPP. Both GGPP and FPP completely prevent pravastatin's stimulation of neurite outgrowth (Fig. 5a). Furthermore, specific inhibition of geranylgeranylation by GGTI-286 mimics the effect of pravastatin by significantly enhancing neurite number and neurite length, relative to the number and length of neurites observed in control cultures (Figs 5b and c). By contrast, the farnesyltransferase inhibitor FTI-277 has no effect on neurite outgrowth (Fig. 5b), which suggests that geranylgeranylated
Fig. 2 Pravastatin significantly increases neurite length and branching. Hippocampal neurons were treated with the HMG-CoA reductase inhibitor pravastatin for 24 h. (a) Pravastatin (100 μM) significantly increased the mean number of branch points per neurite relative to the number of branch points observed in control cells. (b) Pravastatin (50 or 100 μM) also significantly enhanced neurite length. Values represent means ± SEM. *p < 0.05; **p < 0.001 versus control.

Proteins, but not farnesylated proteins, regulate hippocampal neurite outgrowth. We investigated the effect of pravastatin treatment on the Rho-GTPase RhoA, which is activated by translocation to the plasma membrane following geranylgeranylation. Treatment of hippocampal neurons with pravastatin significantly reduces membrane-associated RhoA levels, while increasing levels within the cytosol (Figs 6a and b). Our data suggest that pravastatin promotes neurite outgrowth by a cholesterol-independent mechanism, specifically by preventing geranylgeranylation of RhoA.

In the present study, we determined that the observed effect of pravastatin on neurite outgrowth was not because of cholesterol reduction, as enriching the media with cholesterol did not prevent pravastatin's stimulation of neuritogenesis. Furthermore, treatment of neurons with cholesterol in the absence of pravastatin had no effect on neurite growth, although the concentration of cholesterol used was similar a concentration reported to enhance synaptogenesis (Mauch et al. 2001). Previous studies present conflicting findings: different HMG-CoA reductase inhibitors have been shown to inhibit neurite outgrowth, either by reducing cholesterol levels (Fan et al. 2002) or by inhibition of isoprenylation (Schulz et al. 2004). However, these studies only observed neurite loss after 48 h of statin treatment (either compactin or atorvastatin, respectively), and started treatment within 24 h following plating of primary neuronal cells. In the present study, we grew the neurons in culture for 7 days before starting treatment to better understand the effect that statin treatment might have on more developmentally mature neurons. The neurite loss observed in previous studies may therefore be as a result of different pharmacological profiles of the statins used (pravastatin vs. compactin or atorvastatin), or to increased sensitivity of immature neurons to toxicity.
Fig. 4 Stimulation of neurite outgrowth by pravastatin is blocked by mevalonate but not by cholesterol. (a) Neurons were treated for 24 h with either pravastatin (P), cholesterol (Chol; 20 μg/mL), or pravastatin plus cholesterol. Co-treatment with cholesterol did not prevent pravastatin's stimulation of neuritogenesis; cholesterol alone had no effect on neurite outgrowth. Depletion of cellular cholesterol by methyl-β-cyclodextrin (MβCD; 50 μM; 30-min treatment followed by 24-h recovery in cholesterol-free media) significantly inhibited neurite outgrowth, compared with outgrowth in control cells. (b) Incubation of neurons with mevalonate (Mev; 1 mM) significantly inhibited the stimulatory effect of pravastatin on neurite outgrowth; mevalonate alone had no effect. (c) Mevalonate, but not cholesterol, significantly blocked the increase in neurite length stimulated by pravastatin. Values represent means ± SEM. **p < 0.01, ***p < 0.001; ††p < 0.01 versus control; †††p < 0.001 versus pravastatin-treated group.

Fig. 5 Pravastatin may stimulate neurite outgrowth via inhibition of isoprenoid formation. (a) Co-treatment of hippocampal neurons with pravastatin and geranylgeranylpyrophosphate (GGPP; 20 μM) or farnesylpyrophosphate (FPP; 20 μM) blocked the stimulatory effect of pravastatin on neurite outgrowth. (b) Incubation of neurons with GGTI-286 (50 μM), a highly specific inhibitor of geranylgeranylation, significantly increased neurite outgrowth and neurite length (data not shown), whereas FTI-277 (50 μM), a farnesyltransferase inhibitor, had no effect on either of these measures. Values represent means ± SEM. **p < 0.01, ***p < 0.001 versus control; ††p < 0.01, †††p < 0.001 versus pravastatin-treated group.

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Pravastatin enhances neurite outgrowth in vitro

- **Fig. 6** Pravastatin may stimulate neurite outgrowth via inhibition of Rho isoprenylation. (a) Representative western blots probed with an antibody directed against RhoA. After 4-h pravastatin treatment (100 μM), cell lysates were subjected to subcellular fractionation, and the membrane (top) and cytosol (bottom) fractions separated using SDS-PAGE. C, control; p, pravastatin treated. (b) Four-hour pravastatin treatment (100 μM) significantly prevented association of RhoA with the membrane fraction while increasing RhoA levels in the cytosol, compared with RhoA distribution in control cells. Values represent means ± SEM. *p < 0.05; **p < 0.001 versus control.

induced by inhibition of mevalonate synthesis. Comparison of these results also suggests that statins may either inhibit or stimulate neuronal growth, depending on the stage of neuronal development. Finally, our results are in agreement with earlier findings in PC12 and neuroblastoma cells which suggest that HMG-CoA reductase inhibitors stimulate neurite growth (Maltese and Sheridan 1985; Sato-Suzuki and Murata 1996; Kumano et al. 2000).

Our data indicate that pravastatin’s stimulation of neuritogenesis is because of inhibition of isoprenoid synthesis. Mevalonate is necessary for formation of FPP and its downstream derivative, GGPP. The presence of GGPP is required for the farnesylation of small G-proteins, such as Ras, by farnesyltransferase. Similarly, GGPP is required for geranylgeranylation of the Rho family of proteins by geranylgeranytransferase. Therefore, FPP, GGPP and geranylgeranytransferase, but not farnesyltransferase, are required for the geranylgeranylation of Rho (Noguchi et al. 1998). In the present study, treatment of the neurons with the isoprenoids GGPP and FPP completely prevented the effect of pravastatin on neurite outgrowth. Furthermore, neurite number and neurite length were significantly enhanced by treatment with the geranylgeranytransferase inhibitor GGTI-286, while FTI-277, a farnesyltransferase inhibitor, had no effect on neurite outgrowth; Activation of the Rho family of GTPases requires geranylgeranylation, but not farnesylation (Casey and Seabra 1996). The monomeric G-protein RhoA, in particular, is involved in rearrangement of the actin cytoskeleton; inhibition of RhoA signaling simulates neurite outgrowth in a variety of models (Lee et al. 2000; Scott and Luo 2001; Bryan et al. 2004). Therefore, enhancement of neurite outgrowth by pravastatin is consistent with the hypothesis that reduction of RhoA signaling via inhibition of geranylgeranylation stimulates neuritogenesis.

HMG-CoA reductase inhibitors are either lipophilic or hydrophilic compounds. Lipophilic statins, such as pravastatin, enter cells via an ATP-dependent anion transport system (Nezasa et al. 2003). Pravastatin was not previously thought to cross the blood–brain barrier, even although pravastatin use is associated with a reduced risk of AD (Wolozin et al. 2000; Rockwood et al. 2002). However, it was recently demonstrated in mice that oral pravastatin treatment results in measurable pravastatin levels in the brain (Johnson-Anuna et al. 2005). It will therefore be important to investigate the effects of pravastatin and other lipophilic statins on neuritic growth in vivo, because, as our data suggest, pravastatin may be useful for preventing the neuritic dystrophy caused by AD.

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