Pitavastatin Decreases Tau levels Via the Inactivation of Rho/ROCK

著者	HAMANO Tadanori, YEN Shu-Hui, GENDRON Tania, KO Li-wen, KURIYAMA Masaru
journal or publication title	Neurobiology of Aging
volume	33
year	2011-11
URL	http://hdl.handle.net/10098/4560

title

Pitavastatin Decreases Tau levels Via the Inactivation of Rho/ROCK

Abbreviated title

Pitavastatin Decreases Tau by Inactivation of Rho/ROCK

Tadanori Hamano^{a,*}, Shu-Hui Yen^b, Tania Gendron^b, Li-wen Ko^b, Masaru Kuriyama^a

^a*Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui, Fukui, Japan*

^b*Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, FL, USA*

*Corresponding author at:

Second Department of Internal Medicine, Faculty of Medical Sciences,
University of Fukui, 23-3 Matsuoka shimoaizuki, Eiheiji-cho, Yoshida-gun,
Fukui 910-1193, Japan

Tel.: +81-776-61-3111, Ext 2300, fax: +81-776-61-8110

E-mail address: hamano@u-fukui.ac.jp (T. Hamano)

Number of figures: 12

Number of pages: 25

Number of words for Abstract: 161, Introduction: 423, and Discussion: 853

Abbreviations: AD, Alzheimer's disease; NFT, neurofibrillary tangle; MVA, mevalonate; A β , amyloid

β protein; Tet, tetracycline; GSK3 β , glycogen synthase kinase 3 β

Abstract

Epidemiological studies have shown that long-term treatment with statins decreases the risk of developing Alzheimer's disease. Statins have pleiotropic effects by lowering the concentration of isoprenoid intermediates. Although several studies have shown that statins may reduce amyloid beta protein levels, there have been few reports on the interaction between statins and tau. We report here that pitavastatin reduces total and phosphorylated tau levels in a cellular model of tauopathy, and in primary neuronal cultures. The decrease caused by pitavastatin is reversed by the addition of mevalonate, or geranylgeranyl pyrophosphate. The maturation of small G proteins, including RhoA was disrupted by pitavastatin, as was the activity of glycogen synthase kinase 3 β (GSK3 β), a major tau kinase. Toxin A, inhibitor of glycosylation of small G proteins, and Rho kinase (ROCK) inhibitor decreased phosphorylated tau levels. ROCK inhibitor also inactivated GSK3 β . Although the mechanisms responsible for the reduction in tau protein by pitavastatin require further examination, this report sheds light on possible therapeutic approaches to tauopathy.

Key words: Statin; Pleiotropic effects; Tau; Neuronal cellular model; Small G protein; Rho/ROCK;

Glycogen synthase kinase 3 β

1. Introduction

The accumulation of filamentous inclusions in the central nervous system (CNS) in the form of neurofibrillary tangles (NFTs) is a pathologic hallmark of a group of neurodegenerative disorders, including Alzheimer's disease (AD), progressive supranuclear palsy, corticobasal degeneration, frontotemporal dementia with parkinsonism linked to chromosome 17, and Niemann-Pick disease type C (Lee et al., 2001). NFTs are composed of the microtubule-associated protein tau, which is extensively phosphorylated when incorporated into these inclusions.

There is increasing evidence that disturbances in cholesterol homeostasis play an important role in the pathogenesis of AD (Haley and Dietschy, 2000; Di Paolo and Kim, 2011). Accumulation of free cholesterol in NFT-bearing neurons has been found compared with adjacent NFT-free neurons (Distl et al., 2001, 2003). Cholesterol also accumulates in senile plaques, another major histopathological hallmark of AD (Mori et al., 2001; Panchal et al., 2010).

Hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins), effective for the treatment of hypercholesterolemia, block the production of mevalonate (MVA) (Fig. 1). Statins also exhibit pleiotropic effects by modulating the isoprenylation (farnesylation or geranylation) of various proteins.

It has been reported that long-term treatment of hypercholesterolemia with statins significantly reduces the prevalence of AD (Jick et al., 2000; Wolozin et al., 2007; Haag et al., 2009). Several studies have demonstrated that statin treatment decreases the production of amyloid β protein ($A\beta$), which consists senile plaques (Ostrowski et al., 2007; Abrahamson et al., 2009).

Recently, a transgenic model of tauopathy showed reduction of NFTs by statins in both the early and late stages (Boimel et al., 2009). It was concluded that anti-NFT effects of statins may be related to their anti-inflammatory properties, because microglia was decreased in such brain. Kurata et al. also reported that statins reduced numbers of phosphorylated tau-positive neurites as well as senile

plaques in aged amyloid precursor protein (APP) transgenic mice (2011).

Pitavastatin was chosen in this study, because it is a novel statin with a strong cholesterol-lowering effect, ten times that of simvastatin or pravastatin (Kurata et al., 2010). Another reason is that pitavastatin, unlike other statins, does not affect the control of type 2 diabetes mellitus (Gumprecht et al., 2010) which is another risk factor for AD (Sims-Robinson et al., 2010). To determine the effects and mechanisms of pitavastatin, on tau metabolism, we used transfectant M1C cells, which overexpress human 4R0N tau through a tetracycline off (TetOff)-inducible mechanism. The M1C transfectant cell line was derived from human neuroblastoma BE(2)-M17D cells and previous studies have demonstrated that these cells accumulate sarkosyl-insoluble tau aggregates and oligomeric tau (Ko et al., 2004; Hamano et al., 2008, 2009).

2. Methods

2.1. Materials

Glass-bottom tissue culture dishes were purchased from Mat Tek Corporation (Ashland, MA, USA), and Lab-Tek chambered cover glass was from Nunc (Rochester, NY, USA). Other tissue cultureware was obtained from B.D. Biosciences (Franklin Lakes, NJ, USA). Pitavastatin was kindly provided by Kowa Pharmaceutical Co., Ltd. (Tokyo, Japan). MVA, geranylgeranyl pyrophosphate (GGPP), and *Clostridium difficile* toxin A (Toxin A) were obtained from Sigma (St. Louis, MO, USA). Rho kinase (ROCK) inhibitor, H-1152, was from Calbiochem (La Jolla, CA, USA). Other chemicals were obtained from Sigma, unless otherwise indicated.

2.2. Antibodies

The locations of epitopes recognized by the different anti-tau antibodies used in the present study are shown in Fig. 2A. These antibodies are well characterized and have been used in a number of previous studies (Gamblin et al., 2003; Ko et al., 2004; Hamano et al., 2008; DeTure et al., 2002;

LoPresti et al., 1995; Porzig et al., 2007). The monoclonal antibody Tau5 was obtained from Invitrogen (Carlsbad, CA, USA); TauC3 and anti-RhoA were obtained from Santa Cruz (Santa Cruz, CA, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Rac1 were obtained from Millipore (Billerica, MA, USA); and anti-Cdc42 and anti-flotillin-1 were obtained from BD (San Jose, CA, USA). Monoclonal antibodies to phosphorylated tau protein, PHF-1 and CP13 were obtained from Dr. Peter Davies (Albert Einstein University, Bronx, NY, USA). Tau46 was obtained from Dr. Virginia M.-Y. Lee (University of Pennsylvania, Philadelphia, PA, USA). Polyclonal antibodies to cleaved caspase3, glycogen synthase kinase 3 β (GSK3 β), and phospho-GSK3 β (Ser9) (pGSK3 β) were obtained from Cell Signaling (Danvers, MA, USA), and those to Rac1, Rab5b, and Rab 6 were purchased from Santa Cruz. Antibodies were used at the following dilutions: Tau5 (1:1,000), Tau46 (1:2,000), PHF-1 (1:200), CP13 (1:200), TauC3 (1:2,000), anti-cleaved caspase3 (1:500), anti-GAPDH (1:2,000), anti-RhoA (1:200), anti-Rac1 (1:1,000), anti-Rab4 (1:200), anti-Cdc42 (1:250), anti-flotillin-1(1:1,000), anti-GSK3 β (1:1,000), and anti-pGSK3 β (1:500).

2.3. Cell Culture

M1C cells were seeded at $1.5\text{--}2 \times 10^6$ cells/plate in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, G418 (400 $\mu\text{g/mL}$; Life Technologies, Gaithersburg, MD, USA), and Tet (2 $\mu\text{g/mL}$). Twenty-four hours after seeding, tau expression was elicited by replacing spent medium with fresh medium containing 1 ng/mL Tet (TetOff induction). Replica cultures of M1C cells were exposed to pitavastatin on day 4 of the TetOff induction, and harvested at the end of a day 5 (Fig. 2B). In the experiments on the time course of effects of pitavastatin, M1C cells were exposed to 1 μM of pitavastatin from 0.5, 1, 1.5, 2, or 4 days before harvest at the end of day 5. Other cultures were treated with MVA (250 μM , Sigma) or GGPP (10 μM) for 2 h before being exposed to pitavastatin. It has been shown that 250 μM of MVA effectively reverses the inhibition of mevalonate pathway by statins (Ostrowski et al., 2007), and 10 μM of GGPP effectively reverses the inhibition of

geranylgeranylation by statins (Masamura et al., 2003; Fuchs et al., 2008).

2.4. Primary neuronal cultures

Primary cortical neurons were isolated and purified from female and male mice (Slc:ICR) on embryonic day 16 (E16). Isolated primary neurons were plated onto Lab-Tek chambered coverglass precoated with polyethyleneimine (Sigma) at a density of 5×10^4 per well, or 3.5 cm plastic dish precoated with polyethyleneimine at a density of 4×10^5 per dish for Western blot analysis. The cultures were maintained in serum free Neurobasal medium (Invitrogen) and were treated with 5 μ M cytosine arabinoside (Ara-C) to inhibit proliferation of non neuronal cells (Han et al., 2005). All experiments presented in this work were performed on pure neuronal cells (>95% neuronal purity assessed by immunostaining cultures using neuron specific Anti-NeuN (Millipore)) on 14th day from start plating the cells.

2.5. Fractionation of cell lysates

M1C cells were harvested and homogenized in Tris buffer containing protease and phosphatase inhibitors (30 mM β -glycerophosphate, 30 mM sodium fluoride, and protease inhibitor cocktail (Roche, Germany)), 1 mM EDTA, and 1 mM EGTA. Homogenates were centrifuged at 180g for 15 min to obtain cell lysates. Portions of lysates were further fractionated based on solubility in Tris buffer or 2% sarkosyl to generate SN1, SN2, and S/P fractions, as reported previously (Hamano et al., 2008; Sahara et al., 2002). The SN1 fraction is the supernatant derived from centrifugation of lysates at 150,000g for 15 min at 4 °C. The pellet was re-suspended in buffer containing 0.8 M NaCl, 10% sucrose, 10 mM Tris/HCl (pH 7.4), 1 mM EGTA, protease inhibitor cocktail, and 1% sarkosyl and centrifuged at 150,000g for 15 min to yield a supernatant (SN2) and sarkosyl-insoluble pellet (S/P). All supernatants and the sarkosyl-insoluble pellet re-suspended in Tris buffer were used immediately for immunoblotting, or were stored at -70 °C or -20 °C.

2.6. Western blotting

Cell lysates or fractionated preparations were mixed with Laemmli sample buffer containing 1% β -mercaptoethanol (β ME). Protein concentrations of cell lysates were determined by bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA). Samples containing the same amounts of protein (10 μ g, corresponding to $0.1-0.2 \times 10^5$ cells per lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis and were subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore) for immunoblotting. Blots were immersed in 1% gelatin in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature (RT). After rinsing with TBS-T, blots were incubated with monoclonal antibodies Tau5, PHF-1, CP13, TauC3, anti-flotilin, anti-RhoA, anti-Cdc42, or anti-GAPDH, or polyclonal antibodies to Rab5b, Rab6, Rac1, GSK3 β , pGSK3 β , or cleaved caspase3, for 1 h at RT at the dilutions indicated above. After the third rinse, blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG or goat anti-rabbit IgG for 30 min at RT. After a final wash, immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK) (Hamano et al., 1997). The intensity of immunoreactivity was quantified by capturing images using Photoshop CS version 8.01 (Adobe, San Jose, CA, USA), and analyzed using Image J software (NIH, Bethesda, MA, USA).

2.7. mRNA expression

To examine tau mRNA expression, total RNA was extracted from cells using TRIzol reagent (Invitrogen) and converted to cDNA for PCR amplification. The PCR cycling conditions were: 94 °C, 5 min (1 cycle): 94 °C, 30 s: 50 °C, 30 s: 72 °C, 20 s (29 cycles): 72 °C, 15 min. Primer sequences for tau were 5'-TGAGCCCCGCCAGGAGTTC-3' and 5'-TTGGAGCGGGCGGGGTTTTTG-3', and yielded products of 446 or 355 bp for tau isoforms containing or lacking exon 2 (4R0N), respectively (Gendron et al., 2008). Primer sequences for GAPDH were 5'-TTGATTTTCGAGGGATCTCG-3' and 5'-GAGTCAACGGATTTGGTCGT-3' (product size: 238 bp). Quantitative real time PCR (QPCR) was carried out using 20 ng of cDNA per

sample in a 20 μ l reaction volume, with the StepOnePlus Realtime PCR System. Relative gene expression was calculated using the δ CT methods (Bunpo et al., 2009). All samples were run in triplicate. To analyze Tau mRNA levels, the following TaqMan gene expression assays (Applied Biosystems) were used: MAPT (Hs00902188_m1) and GAPDH (Hs99999905_m1).

2.8. Morphological study

Cells were cultured on 24-well plates, and morphological studies before and after pitavastatin treatment were performed using an inverted microscope (IX-70; Olympus, Tokyo, Japan). Images were captured by a digital camera (DP-70; Olympus). Dead cell counts were estimated by ATP assay using The CellTiter-Glo Luminescent Cell Viability Assay Kit, according to the manufacturer's instructions.

2.9. Immunocytochemical study

Cells grown on glass-bottom culture dishes were subjected to TetOff induction in the absence or presence of pitavastatin (2 μ M) for the final 24 h of induction. After treatment, cells were washed with phosphate-buffered saline (PBS), permeabilized with 0.0001% saponin/PBS, and exposed to methanol at -20 $^{\circ}$ C prior to fixation in 2% paraformaldehyde/PBS. Fixed samples were rinsed with 100 mM Tris-saline (TS), blocked with TS containing 3% goat serum, and then incubated with P44 antibody (1:100) followed by Alexa 594 anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) and Alexa 488 anti-mouse IgG (Molecular Probes). A confocal fluorescence microscope (TCS SP II; Leica, Heidelberg, Germany) was used to visualize immunoreactivity.

2.10. Membrane localization and Western blotting for GTPases

M1C cells were treated with pitavastatin for 24 h. Cellular fractionation was carried out as described by Zhao et al. (2002). Briefly, following pitavastatin treatment, 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 10 s sonication. Cells were removed by

centrifugation at 500g for 5 min at 4 °C. The resulting supernatant was removed (cytosolic fraction), and the membrane pellets were resuspended in relaxation buffer (membrane fraction).

2.11. Immunoblotting of the inactive form of GSK3 β

GSK3 β was analyzed by comparing the phosphorylation levels of GSK3 β (Ser9) before and after pitavastatin treatment, according to the method described by Ma et al. (2009).

2.12. Toxin A and Rho kinase (ROCK) inhibitor assay

The effects of Toxin A were analyzed by treating cells with 500 ng/mL of Toxin A or 1 μ M of ROCK inhibitor for the final 15 h of the 5-day TetOff induction period. The total amount of tau protein was determined by Western blotting before and after Toxin A or ROCK inhibitor treatment.

2.13. Statistical analysis

All values in the figures are expressed as the mean $\pm$ SEM. Differences between groups were analyzed by Student's *t*-test (SPSS version 17.0), with findings of $p < 0.05$ considered significant. IC₅₀ values were calculated by non linear regression.

3. Results

3.1. Pitavastatin dose-dependently decreases tau levels and inhibits tau phosphorylation

To examine the effect of pitavastatin on tau, M1C cells were induced to express tau for 5 days and exposed to pitavastatin (0.5, 1, 2, 5, or 10 μ M) during the final day of the induction period. Control cultures were treated with DMSO, the vehicle used to dilute pitavastatin. Lysates derived from cultures were analyzed by Western blot using antibody Tau5 or Tau46. These samples were also probed with anti-GAPDH antibody to verify that loading among lanes was equal (Fig. 3). Total tau levels were decreased in a dose-dependent fashion following pitavastatin treatment, except for that at the highest dose examined (10 μ M). At this dose, a ~50 kDa tau species became evident. In cultures treated with 1 μ M pitavastatin, antibody Tau5 detected 45- to 62-kDa bands at $68 \pm 17.3\%$ of

the level displayed in the vehicle control (mean $\pm$ SEM) and antibody Tau46 detected 50- to 58-kDa bands at $62.3 \pm 10.6\%$ of the level displayed in the vehicle control.

To determine whether this decrease in tau was the result of changes in tau mRNA levels, mRNA expression was examined. As shown in Fig. 5, pitavastatin had no effect on tau mRNA levels, as determined by semiquantitative RT-PCR (A), as well as QPCR (B).

Next, phosphorylated tau in lysates was assessed using phospho-tau antibodies that recognize 4 epitopes collectively (Fig. 2A), and blots were subsequently reprobed using GAPDH and Tau5, Tau46, or P44 antibodies. In cultures treated with 1 μ M pitavastatin, antibodies PHF-1 and CP13 detected 52- to 68-kDa bands at $58 \pm 5.4\%$ and $40.1 \pm 7.2\%$, respectively, of the level displayed in the vehicle control (Fig. 3).

In the time course experiments, 1.5 days (36 h) of 1 μ M pitavastatin treatment most effectively decreased total tau levels, as well as phosphorylated tau levels (Fig. 4).

Of note, pitavastatin treatment decreased the ratio of phosphorylated tau to total tau (PHF-1/Tau5 or CP13/Tau5) (Fig. 6A). However, the level of Ser9-phosphorylated GSK3 β increased following pitavastatin treatment (Fig. 6B), indicating that GSK3 β was inactivated by pitavastatin. Given that GSK3 β is known to phosphorylate tau, these findings suggest that pitavastatin may decrease levels of phosphorylated tau, at least in part, by inhibiting GSK3 β .

3.2. Pitavastatin decreases the accumulation of sarkosyl-insoluble tau in M1C cells

To assess whether pitavastatin causes the clearance of soluble versus insoluble tau, M1C cells were induced to express tau for 5 days and exposed to 0.5 μ M or 2 μ M of pitavastatin on day 4. Cell lysates were then fractionated to derive SN1 (buffer soluble), SN2 (salt- and sarkosyl-soluble), and S/P (sarkosyl-insoluble pellet) fractions. A portion of each sample was analyzed by Western blot using antibody Tau5 (Fig. 7). Total tau levels, including those in the sarkosyl-insoluble fraction, were decreased by 2 μ M, as well as 0.5 μ M pitavastatin.

3.3 Pitavastatin decreases endogenous tau, as well as phosphorylated tau, in primary neurons

To test the effect of pitavastatin on endogenous tau, primary neuronal cultures were treated with pitavastatin (0.2, 1 and 5 μM). This led to a reduction in total tau levels, and in levels of phosphorylated tau in a dose-dependent manner, as assessed by Western blotting. ATP levels did not change after treatment with 0.5, 1, or 5 μM of the statin. The IC_{50} value of pitavastatin obtained from the ATP assay was 120 μM for neuronal cells (Fig. 8A). A reduction of phosphorylated tau (CP13) following pitavastatin treatment was also observed by immunocytochemical analysis (Fig. 8B).

3.4. Treating cells with low-to-moderate concentrations of pitavastatin does not cause cell death

To examine the effect of pitavastatin on cell morphology, M1C cells were cultured on 24-well plates, and examined by phase-contrast microscopy (Fig. 9A). There were no obvious differences between cultures treated with vehicle and pitavastatin at concentrations ranging from 0.5-5 μM . However, high-dose pitavastatin (10 μM) induced alterations of cell body morphology suggestive of apoptosis.

Next, we evaluated whether pitavastatin induced cell death. Low-to-moderate dose pitavastatin (0.1-5 μM) did not further reduce the number of living cells, but high-dose pitavastatin (10 μM) reduced the number by about 30% (Fig. 9B). IC_{50} value obtained from the ATP assay was 140 μM pitavastatin for M1C cells. Congruent with these findings, cleaved caspase 3 was not detected following treatment with low-dose pitavastatin (0.5-2 μM). A weak band of cleaved caspase 3 was detected after treatment with 5 μM pitavastatin, while high concentrations of pitavastatin (10 μM) caused caspase activation (Fig. 9C). Interestingly, this activation correlated with the emergence of caspase-cleaved tau, as detected by TauC3 (Fig. 9D).

3.5. Reduction of tau by pitavastatin treatment is reversed by the addition of mevalonate

By inhibiting HMG-CoA reductase, statins also diminish the production of MVA and isoprenoids (Fig. 1). To determine whether exogenous MVA could prevent reduction in tau observed in cells

treated by pitavastatin, M1C cells induced to express tau were co-treated with 2 μ M pitavastatin and MVA (250 μ M). As shown in Fig. 10A and 10B, MVA reversed the decrease in tau normally caused by pitavastatin treatment, suggesting that this decrease was not a toxic effect of pitavastatin, but instead occurred as a result of the decreased production of isoprenoids. This is further supported by the fact that GGPP also reversed pitavastatin-induced tau clearance, showing that this effect was dependent on the inhibition of protein geranylgeranylation (Fig. 10C).

3.6. Pitavastatin treatment decreased membrane-associated small G proteins

Geranylgeranylation of small G proteins of the Rho family is an essential prerequisite for their anchoring in the cell membrane and thus for their activity. Given that pitavastatin decreased tau levels and given that this effect was blocked by GGPP, we examined whether pitavastatin influenced the cellular localization of small G proteins. Pitavastatin treatment decreased levels of membrane-associated small G proteins, including RhoA, Rac1, Cdc42, Rab1B, Rab4, Rab5, and Rab6, while increasing levels of these proteins in the cytosolic fraction. MVA reversed the decrease in membrane-associated small G proteins (Fig. 11).

3.7. Toxin A and Rho kinase (ROCK) inhibitor treatment decreased tau levels

Toxin A is a robust and specific Rho family inhibitor often used to delineate the Rho family-dependent effects of statins (Voth and Ballard, 2005). Toxin A treatment (500 ng/mL) decreased total tau levels (Fig. 11A). ROCK inhibitor (1 μ M) also decreased total and phosphorylated tau (Fig. 12B). Of interest, treatment of cells with ROCK inhibitor led to the inactivation of GSK3 β by increasing GSK3 β phosphorylation at Ser9 (Fig. 11C). These findings were replicated using a second ROCK inhibitor (Y-27632, Wako, Osaka, Japan) (data not shown). IC₅₀ values obtained by ATP assay were 1,630 ng/mL (Toxin A), and 84 μ M (ROCK inhibitor) for M1C cells.

4. Discussion

Recently, interest in the therapeutic potential of statins for the treatment of AD has developed. Indeed, some evidence of their beneficial effects in mild to moderate AD has been reported (Jick et al., 2000; Wolozin et al., 2000; Rockwood et al., 2002; Hajjar et al., 2002; Zamrini et al., 2004; Dufouil et al., 2005). Furthermore, epidemiological studies have shown that hypercholesterolemia is a risk factor for AD (Haley and Dietschy, 2000; Di Paolo and Kim, 2011). A randomized controlled trial of atorvastatin in mild to moderate AD, however, was not associated with significant clinical benefit (Feldman et al., 2010).

We have shown herein that pitavastatin treatment markedly reduces tau levels, as well as tau phosphorylation, in both a cellular model of tauopathy and in primary neurons. This is in agreement with a study conducted by Boimel et al. (2009), who reported that statins reduce NFTs in a model of tauopathy. They showed that treatment (1 month) with simvastatin or atorvastatin reduced both NFT burden and lectin-positive microglia. Simvastatin also improved memory deficits, as evaluated by T-maze performance, in young mice treated for 8 months. Kurata et al. also reported that statin (atorvastatin and pitavastatin) reduced the numbers of senile plaques and tau positive neuritis in APP transgenic mice (2011).

Of particular interest, recent clinical observations have shown that simvastatin, a CNS-permeable statin, decreases the amount of phosphorylated tau in the cerebrospinal fluid of AD patients (Riekse et al., 2006). Kurzepa et al. (2008) also reported that administration of simvastatin following stroke prevents increase in the serum levels of tau protein.

Nonetheless, it must be indicated that, despite the cholesterol lowering effects of statins, the role of cholesterol in tau metabolism remains controversial. Fan et al. (2001) reported that cholesterol deficiency induced by compactin in cultured neurons results in hyperphosphorylation of tau accompanied by axonal degeneration associated with microtubule depolymerization. Meske et al.

(2003) also reported that lovastatin treatment transiently increases tau phosphorylation, causing alterations in the microtubule system in a rat primary neuron culture model.

In our study, we found the effects of pitavastatin on tau to be dose-dependent. Low-to-moderate doses of pitavastatin, which did not activate caspase, reduced total and phosphorylated tau levels. In contrast, high dose pitavastatin activated caspase 3 and increased levels of caspase-cleaved tau, which may facilitate tau aggregation. We also found that a higher dose of pitavastatin treatment caused morphological changes suggestive of apoptosis (Fig. 9A). These findings are similar to the results of a study by Meske et al. (2003). However, Bar-On et al. (2008) reported that 10 μ M statin (lovastatin, simvastatin, or pravastatin) treatment for 24 h did not cause cell death in B103 neuroblastoma cells. These inconsistencies in findings may be due to the different types of cells, statins and concentration used among studies.

Statins exhibit pleiotropic effects through their ability to lower the concentration of isoprenoid intermediates, such as GGPP or FPP. Members of the Rho and Rab family of small G proteins require the addition of these isoprenyl moieties at their C-terminus for acquisition of normal GTPase function. The Rho subfamily of small G proteins, which include RhoA, Rac, and Cdc42, was first recognized for their regulatory effects on cytoskeletal rearrangement (Nobes and Hall, 1995). The findings of the present study suggest that inhibition of small G protein maturation may participate in the reduction of tau levels, since the addition of MVA or GGPP blocked pitavastatin-induced tau clearance. In support of this, pitavastatin treatment led to altered membrane distribution of small G proteins, including RhoA, Rac1, Rab5, and Rab6 proteins (Fig. 11). Interestingly, Eckert et al. (2009) recently reported that FPP and GGPP levels in brains of AD patients are upregulated compared with that in those of healthy individuals, and that, following simvastatin treatment, these levels are downregulated. Scheper et al. (2007) also reported that Rab6 is upregulated in AD brain and that it colocalizes with hyperphosphorylated tau.

It has been reported that Galpha13 activation by lysophotidic acid (LPA) results in RhoA activation. This RhoA activation activates GSK3 β (Sayas et al., 2002). We speculated that the decrease in phosphorylated tau by pitavastatin treatment was caused by GSK3 β inactivation through RhoA inactivation. GSK3 β inactivation was observed following low-dose pitavastatin treatment (Fig. 5B), consistent with the findings of Ma et al. (2009). Rho-GTPase activates the Rho kinases, ROCK1/2. Rho-GTPase, ROCK1/2, and PAK1-3 are important regulators of synaptic plasticity especially in maintaining the actin cytoskeleton of dendritic spines. Recently, it was suggested that RhoA/ROCK acts through myosinII to destabilize microtubules (Takesono et al., 2010).

We found that ROCK inhibitor led to GSK3 β inhibition by increasing its phosphorylation, and also decreased the levels phosphorylated tau (Fig. 12B, C). Further evaluation is needed on the interaction between tau and Rho/ROCK, and the induction of NFT formation by microtubule disturbances and Rho/ROCK signaling.

Overall, our findings provide a insight into how statins may reduce total and phosphorylated tau levels in AD patients and highlight the implications of Rho/ROCK for the metabolism including phosphorylation of tau. Although the mechanisms responsible for the reduction in tau protein by pitavastatin require further examination, this report sheds light on possible therapeutic approaches to tauopathy.

Disclosure statement

Competing Interest: None

Acknowledgements

A part of this study was supported by Research Grant from University of Fukui. We are grateful to Miss Haruna Kusaka, Chiemi Ishida, and Tomomi Kame for their technical help in this study, as

well as Drs. Min-Jue Xie (Department of Anatomy, University of Fukui), Masahiro Ohtani and Yoshiyuki Ito (Second Department of Internal Medicine, University of Fukui) for their technical advice regarding neuron culture and mRNA study. We are also grateful to Kowa Pharmaceutical Co., Ltd., for kindly providing pitavastatin, Dr. Peter Davies (Albert Einstein University, Bronx, NY, USA) for providing PHF-1 and CP13 antibodies, and to V. M. Lee (University of Pennsylvania, PA, USA) for the generous gift of Tau46 antibody.

References

- Abrahamson, E.E., Ikonomic, M.D., Dixon, C.E., DeKosky, S.T., 2009. Simvastatin therapy prevents brain trauma-induced increases in beta-amyloid peptide levels. *Ann. Neurol.* 66, 407-414.
- Bar-On, P., Crews, L., Koob, A., Mizuno, H., Adame, A., Spencer, B., Masliah, E., 2008. Statins reduce neuronal alpha-synuclein aggregation in in vitro models of Parkinson's disease. *J. Neurochem.* 105, 1656-1667.
- Boimel, M., Grigoriadis, N., Lourdopoulos, A., Touloumi, O., Rosenmann, D., Abramsky, O., Rosenmann, H., 2009. Statins reduce the neurofibrillary tangle burden in a mouse model of tauopathy. *J. Neuropathol. Exp. Neurol.* 68, 314-325.
- Bunpo, P., Dudley, A., Cundiff, J.K., Cavener, D.R., Wek, R.C., Anthony, T.G. 2009. GCN2 protein kinase is required to activate amino acid deprivation responses in mice treated with the anti-cancer agent L-asparaginase. *J. Biol. Chem.* 284, 32742-32749.
- DeTure, M., Ko, L.W., Easson, C., Yen, S.H., 2002. Tau assembly in inducible transfectants expressing wild-type or FTDP-17 tau. *Am. J. Pathol.* 161, 1711-1722.
- Di Paolo G, Kim TW., 2011. Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat. Rev. Neurosci.* 12, 284-296.

- Distl, R., Meske, V., Ohm, T.G., 2001. Tangle-bearing neurons contain more free cholesterol than adjacent tangle-free neurons. *Acta. Neuropathol.* 101, 547-554.
- Distl, R., Treiber-Held, S., Albert, F., Meske, V., Harzer, K., Ohm, T.G., 2003. Cholesterol storage and tau pathology in Niemann-Pick type C disease in the brain. *J. Pathol.* 200, 104-111.
- Dufouil, C., Richard, F., Fiévet, N., Dartigues, J.F., Ritchie, K., Tzourio, C., Amouyel, P., Alperovitch, A., 2005. APOE genotype, cholesterol level, lipid-lowering treatment, and dementia: the Three-City Study. *Neurology* 64, 1531-1538.
- Eckert, G.P., Hooff, G.P., Strandjord, D.M., Igbavboa, U., Volmer, D.A., Müller, W.E., Wood, W.G., 2009. Regulation of the brain isoprenoids farnesyl- and geranylgeranylpyrophosphate is altered in male Alzheimer patients. *Neurobiol. Dis.* 35, 251-257.
- Fan, Q.-W., Yu, W., Senda, T., Yanagisawa, K., Michikawa, M., 2001. Cholesterol-dependent modulation of tau phosphorylation in cultured neurons. *J. Neurochem.* 76, 391-400.
- Feldman, H.H., Doody, R.S., Kivipelto, M., Sparks, D.L., Waters, D.D., Jones, R.W., Schwam, E., Schindler, R., Hey-Hadavi, J., DeMicco, D.A., Breazna, A.; LEADe Investigators, 2010. Randomized controlled trial of atorvastatin in mild to moderate Alzheimer disease: LEADe. *Neurology* 74, 956-964.
- Fuchs D, Berges C, Opelz G, Daniel V, Naujokat C. 2008. HMG-CoA reductase inhibitor simvastatin overcomes bortezomib-induced apoptosis resistance by disrupting a geranylgeranyl pyrophosphate-dependent survival pathway. *Biochem. Biophys. Res. Commun.* 374: 309-314.
- Gamblin, T.C., Chen, F., Zambrano, A., Abraha, A., Lagalwar, S., Guillozet, A.L., Lu, M., Fu, Y., Garcia-Sierra, F., LaPointe, N., Miller, R., Berry, R.W., Binder, L.I., 2003. Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10032-10037.
- Gendron, T.F., McCartney, S., Causevic, E., Ko, L.W., Yen, S.H., 2008. Ethanol enhances tau

- accumulation in neuroblastoma cells that inducibly express tau. *Neurosci. Lett.* 443, 67-71.
- Gumprecht, J, Gosho, M, Budinski, D, Hounslow, N., 2011. Comparative long-term efficacy and tolerability of pitavastatin 4 mg and atorvastatin 20-40 mg in patients with type 2 diabetes mellitus and combined (mixed) dyslipidaemia. *Diabetes Obes. Metab.* doi: 10.1111/j.1463-1326.
- Haag, M.D., Hofman, A., Koudstaal, P.J., Stricker, B.H., Breteler, M.M., 2009. Statins are associated with a reduced risk of Alzheimer disease regardless of lipophilicity. The Rotterdam Study. *J. Neurol. Neurosurg. Psychiatry* 80, 13-17.
- Hajjar, I., Schumpert, J., Hirth, V., Wieland, D., Eleazer, G.P., 2002. The impact of the use of statins on the prevalence of dementia and the progression of cognitive impairment. *J. Gerontol. A. Biol. Sci. Med. Sci.* 57, M414-418.
- Haley, R.W., Dietschy, J.M., 2000. Is there a connection between the concentration of cholesterol circulating in plasma and the rate of neuritic plaque formation in Alzheimer disease? *Arch. Neurol.* 57, 1410-1412.
- Hamano, T., Yoshimura, M., Yamazaki, T., Shinkai, Y., Yanagisawa, K., Kuriyama, M., Ihara, Y., 1997. Amyloid beta-protein (A beta) accumulation in the leptomeninges during aging and in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 56, 922-932.
- Hamano, T., Gendron, T.F., Causevic, E., Yen, S.H., Lin, W.L., Isidoro, C., DeTure, M., Ko, L.W., 2008. Autophagic-lysosomal perturbation enhances tau aggregation in transfectants with induced wild-type tau expression. *Eur. J. Neurosci.* 27, 1119-1130.
- Hamano, T., Gendron, T.F., Ko, L.W., Yen, S.H., 2009. Concentration-dependent effects of proteasomal inhibition on tau processing in a cellular model of tauopathy. *Int. J. Clin. Exp. Pathol.* 2, 561-573.
- Han, P., Dou, F., Li, F., Zhang, X., Zhang, Y.-W., Zheng, H., Lipton, S.A., Xu, H., Liao, F.-F., 2005. Suppression of cyclin-dependent kinase 5 activation by amyloid precursor protein: a novel

- excitoprotective mechanism involving modulation of tau phosphorylation. *J. Neurosci.* 25, 11542-11552.
- Jick, H., Zornberg, G.L., Jick, S.S., Seshadri, S., Drachman, D.A., 2000. Statins and the risk of dementia. *Lancet* 356, 1627-1631.
- Ko, L.W., Rush, T., Sahara, N., Kersh, J.S., Easson, C., Deture, M., Lin, W.L., Connor, Y.D., Yen, S.H., 2004. Assembly of filamentous tau aggregates in human neuronal cells. *J. Alzheimers. Dis.* 6, 605-622.
- Kurata, T., Miyazaki, K., Kozuki, M., Panin, V.L., Morimoto, N., Ohta, Y., Nagai, M., Ikeda, Y., Matsuura, T., Abe, K., 2011. Atorvastatin and pitavastatin improve cognitive function and reduce senile plaque and phosphorylated tau in aged APP mice. *Brain Res.* 1371, 161-170.
- Kurzepa, J., Bielewicz, J., Bartosik-Psujek, H., Szczepańska-Szerej, A., Stelmasiak, Z., 2008. Simvastatin inhibits the increase in serum tau protein levels in the acute phase of ischemic stroke. *Pharmacol. Rep.* 60, 1014-1018.
- Lee, V.M., Goedert, M., Trojanowski, J.Q., 2001. Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24, 1121-1159.
- LoPresti, P., Szuchet, S., Papasozomenos, S.C., Zinkowski, R.P., Binder, L.I., 1995. Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes. *Proc. Natl. Acad. Sci. U.S.A.* 92, 10369-10373.
- Ma, T., Zhao, Y., Kwak, Y.D., Yang, Z., Thompson, R., Luo, Z., Xu, H., Liao, F.F., 2009. Statin's excitoprotection is mediated by sAPP and the subsequent attenuation of calpain-induced truncation events, likely via rho-ROCK signaling. *J. Neurosci.* 29, 11226-11236.

Masamura K, Oida K, Kanehara H, Suzuki J, Horie S, Ishii H, Miyamori I. 2003.

Pitavastatin-induced thrombomodulin expression by endothelial cells acts via inhibition of small G proteins of the Rho family. *Arterioscler. Thromb. Vasc. Biol.* 23, 512-517.

Meske, V., Albert, F., Richter, D., Schwarze, J., Ohm, T.G., 2003. Blockade of HMG-CoA reductase activity causes changes in microtubule-stabilizing protein tau via suppression of geranylgeranylpyrophosphate formation: implications for Alzheimer's disease. *Eur. J. Neurosci.* 17, 93-102.

Mori, T., Paris, D., Town, T., Rojiani, A.M., Sparks, D.L., Delledonne, A., Crawford, F., Abdullah, L.I., Humphrey, J.A., Dickson, D.W., Mullan, M.J., 2001. Cholesterol accumulates in senile plaques of Alzheimer disease patients and in transgenic APP(SW) mice. *J. Neuropathol. Exp. Neurol.* 60, 778-785.

Nobes, C.D., Hall, A., 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53-62.

Ostrowski, S.M., Wilkinson, B.L., Golde, T.E., Landreth, G., 2007. Statins reduce amyloid-beta production through inhibition of protein isoprenylation. *J. Biol. Chem.* 282, 26832-26844.

Panchal, M., Loeper, J., Cossec, J.C., Perruchini, C., Lazar, A., Pompon, D., Duyckaerts, C. 2010. Enrichment of cholesterol in microdissected Alzheimer's disease senile plaques as assessed by mass spectrometry. *J. Lipid Res.* 51, 598-605.

Porzig, R., Singer, D., Hoffmann, R., 2007. Epitope mapping of mAbs AT8 and Tau5 directed against hyperphosphorylated regions of the human tau protein. *Biochem. Biophys. Res. Commun.* 358, 644-649.

- Riekse, R.G., Li, G., Petrie, E.C., Leverenz, J.B., Vavrek, D., Vuletic, S., Albers, J.J., Montine, T.J., Lee, V.M., Lee, M., Seubert, P., Galasko, D., Schellenberg, G.D., Hazzard, W.R., Peskind, E.R., 2006. Effect of statins on Alzheimer's disease biomarkers in cerebrospinal fluid. *J. Alzheimers Dis.* 10, 399-406.
- Rockwood, K., Kirkland, S., Hogan, D.B., Macknight, C., Merry, H., Verreault, R., Wolfson, C., McDowell, I., 2002. Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. *Arch. Neurol.* 59, 223-227.
- Sahara, N., Lewis, J., DeTure, M., McGowan, E., Dickson, D.W., Hutton, M., Yen, S.H., 2002. Assembly of tau in transgenic animals expressing P301L tau: alteration of phosphorylation and solubility. *J. Neurochem.* 83, 1498-1508.
- Sayas, C.L., Avila, J., Wandosell, F., 2002. Glycogen synthase kinase-3 is activated in neuronal cells by Galpha12 and Galpha13 by Rho-independent and Rho-dependent mechanisms. *J. Neurosci.* 22, 6863-6875.
- Scheper, W., Hoozemans, J.J., Hoogenraad, C.C., Rosemuller, A.J., Eikelenboom, P., Baas, F., 2007. Rab6 is increased in Alzheimer's disease brain and correlates with endoplasmic reticulum stress. *Neuropathol. Appl. Neurobiol.* 33, 523-532.
- Sims-Robinson, C., Kim, B., Rosko, A., Feldman, E.L., 2010. How does diabetes accelerate Alzheimer disease pathology? *Nat. Rev. Neurol.* 6, 551-559.
- Takesono, A., Heasman, S.J., Wojciak-Stothard, B., Garg, R., Ridley, A.J., 2010. Microtubules regulate migratory polarity through Rho/ROCK signaling in T cells. *PLoS One* 5, e8774.
- Voth, D.E., Ballard, J.D., 2005. Clostridium difficile toxins: mechanism of action and role in disease. *Clin. Microbiol. Rev.* 18, 247-263.
- Wolozin, B., Wang, S.W., Li, N.C., Lee, A., Lee, T.A., Kazis, L.E., 2007. Simvastatin is associated

with a reduced incidence of dementia and Parkinson's disease. *B.M.C. Med.* 5, 20.

Zamrini, E., McGwin, G., Roseman, J.M., 2004. Association between statin use and Alzheimer's disease. *Neuroepidemiology* 23, 94-98.

Zhao, X., Bey, E.A., Wientjes, F.B., Cathcart, M.K., 2002. Cytosolic phospholipase A2 (cPLA2) regulation of human monocyte NADPH oxidase activity. cPLA2 affects translocation but not phosphorylation of p67(phox) and p47(phox). *J. Biol. Chem.* 277, 25385-25392.

Figure legends

Fig. 1. Mevalonate cascade and its inhibitors. Statins inhibit HMG-CoA reductase and block the synthesis of isoprenoids (FPP/GGPP) and cholesterol. Post-translational geranylgeranylation and farnesylation of small G proteins of the Rho/Rab family are required for these proteins to become anchored to the cell membrane, and thus become active. FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

Fig. 2. (A) Schematic representation of 4R0N tau and distinct epitopes recognized by different tau antibodies. (B) Treatment schedule of tetracycline off (TetOff) induction. On day 0, TetOff induction with initiated by decreasing the concentration of tetracycline in the medium from 2,000 ng/mL to 1 ng/mL. On day 4, pitavastatin treatment was initiated. On day 5, cells were harvested.

Fig. 3. Dose-dependent effects of pitavastatin treatment on tau.

M1C cells were subjected to a 5-day TetOff induction of tau expression and cells were exposed to pitavastatin (statin) at 0.5, 1, 2, 5, or 10 μ M during the final day of induction, 1 day before the end of induction. Cultures treated with DMSO served as vehicle controls (0 μ M). Lysates derived from cultures were immunoblotted with antibodies Tau5 (A), Tau46 (B), PHF-1 (C), and CP13 (D). GAPDH was used as an internal standard. (A) The amount of Tau-5-immunopositive tau decreased in a dose-dependent fashion when cells were treated with pitavastatin at low to moderate

concentrations (0.5-5 μ M). However, at the highest doses tested (10 μ M), a slight increase in the amount of tau was noted compared to tau levels in cultures treated with 5 μ M of pitavastatin. (B) Bands immunopositive for Tau46, which recognizes the C-terminus of tau, were also decreased. When the cells were treated in response to 10 μ M statin, the amount of tau did not increase, suggesting that the Tau5-immunoreactive tau species that increased with 10 μ M pitavastatin treatment was a C-terminus-truncated product of tau. (C) Phosphorylated tau detected by PHF-1 and by CP13 (D) were also decreased. However, 10 μ M statin treatment slightly increased the amount of phosphorylated tau detected by PHF-1. Bar: $\pm$ SEM, * p <0.05, ** p <0.01. N=5.

Fig.4. Time course of the effects of pitavastatin on tau levels.

Time course experiments showed that 1.5 days (36 h) of pitavastatin treatment most effectively reduced total and phosphorylated tau protein levels. Bar: $\pm$ SEM, * p <0.05, ** p <0.01. N=5.

Fig. 5. Pitavastatin treatment does not alter tau mRNA levels.

Tau mRNA levels were examined after pitavastatin (statin) treatment by reverse transcriptase PCR (A), and quantitative real-time PCR (B). The statin was found to have no effect on tau mRNA expression. Bar: $\pm$ SEM, ** p <0.01, NS: not significant. N=6. NI: non-induced cells.

Fig. 6. Pitavastatin decreases tau phosphorylation by inhibiting GSK3 β .

(A) The ratio of phosphorylated to total tau was decreased following pitavastatin (statin) treatment (PHF-1/Tau5 or CP13/Tau5). (B) Statin treatment increased GSK3 β phosphorylation at Ser9, which renders GSK3 β inactive. Bar: $\pm$ SEM, * p <0.05. N= 5.

Fig. 7. Pitavastatin suppresses accumulation of sarkosyl-insoluble tau in M1C cells.

Lysates from M1C cells induced to express tau for 5 days were exposed to 0.5 μ M or 2 μ M pitavastatin (statin) (A) on day 4, and then fractionated into SN1 (buffer soluble), SN2 (salt- and sarkosyl-soluble), and S/P (sarkosyl-insoluble pellet) fractions. Each fraction was analyzed by Western blotting using Tau5 antibody. Compared to that in vehicle-treated cells, the amount of tau

was decreased in all fractions obtained from cells treated with 2 μ M as well as 0.5 μ M pitavastatin.

Fig. 8. A. Pitavastatin lowers endogenous tau levels in primary neuronal cultures.

To examine the effect on endogenous tau, primary neurons were treated with pitavastatin (0.2, 1, or 5 μ M) for 24 h. (A) Pitavastatin caused a reduction in total tau, as well as phosphorylated tau (PHF-1, and CP13) levels, as assessed by Western blotting. The IC_{50} value obtained from the ATP assay was 120 μ M for primary neurons. (B) Immunocytochemical analysis also showed a reduction of phosphorylated tau protein (CP13) by 0.5 μ M of statin. Bar: 10 μ m.

Bar: $\pm$ SEM, * P <0.05, ** P <0.01

Fig. 9. High-dose pitavastatin causes morphological changes and cell death.

(A) High-dose pitavastatin (statin) (10 μ M) treatment induced morphological changes including shrinking of the cell body and dendritic processes. However, low to moderate doses of pitavastatin (<5 μ M) did not result in marked morphological changes compared to untreated cells. (B) ATP assays demonstrated that the treatment of tau-expressing cells with low to moderate doses (0.1-5 μ M) of pitavastatin did not affect viability. In contrast, treatment with 10 μ M of pitavastatin decreased the number of live cells. (C) High-dose statin activates caspase 3 and induces caspase cleavage of tau. Cleaved caspase 3, indicative of active caspase 3, was not detected in cells treated with 0-2 μ M statin; however, weak bands were detected after 5 μ M treatment, and stronger bands were detected after 10 μ M treatment. The IC_{50} value of pitavastatin obtained from ATP assays was 140 μ M for M1C cells. (D) Cells treated with 10 μ M statin were immunopositive for TauC3, which detects caspase-cleaved tau. NI: non-induced cells.

Fig. 10. Mevalonate (MVA) and geranylgeranyl pyrophosphate (GGPP) reversed pitavastatin-induced decreases in tau levels.

(A) The decrease in tau caused by pitavastatin (statin) treatment was reversed by co-treatment with 250 μ M MVA. (B) Immunocytochemical analysis of cells demonstrated that non-induced cells

exhibited no tau accumulation (NI), while TetOff-induced cells (DMSO) exhibited tau expression. After treatment of cells with 2 μ M statin, tau accumulation was reduced. However, in cells co-treated with statin and MVA (statin + MVA), the immunoreactivity for tau was recovered. (C) The reduction of tau by statin treatment was also reversed by co-treating cells with 10 μ M GGPP. Statin: 2 μ M pitavastatin, MVA: 250 μ M MVA, GGPP: 10 μ M GGPP.

Fig. 11. Pitavastatin inhibits small G protein membrane association.

After 2 μ M pitavastatin (statin) treatment, the distribution of small G proteins (RhoA, Rac1, Cdc42, Rab1B, Rab4, Rab5 and Rab6) shifted from the membrane fraction to the cytosolic fraction. This was not observed when cells were co-treated with both statin and mevalonate (MVA). Flotilin was used as a marker for the membrane fraction, whereas GAPDH was a marker for the cytoplasmic fraction.

Fig. 12. *Clostridium difficile* Toxin A (Toxin A) and a Rho kinase (ROCK) inhibitor decreased tau.

(A) The relationship between reduction in tau and the inactivation of geranylated Rho family proteins (RhoA, Rac, and Cdc42) was examined by treating cells with 500 ng/mL of Toxin A. Toxin A treatment reduced total tau protein levels. The IC_{50} value of Toxin A obtained from ATP assays was 1,630 ng/mL. -: DMSO control, +: Toxin A, cells treated with 500 ng/mL Toxin A. (B) The ROCK inhibitor (1 μ M) decreased total (Tau5) and phosphorylated (PHF-1 and CP13) tau levels. The IC_{50} value of ROCK inhibitor obtained from ATP assays was 84 μ M. (C) The ROCK inhibitor (1 μ M) inactivated GSK3 β by increasing its phosphorylation at Ser9. -: DMSO control, +: cells treated with 1 μ M ROCK inhibitor.