Statins inhibit the dimerization of β -secretase via both isoprenoid- and cholesterol-mediated mechanisms

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We have previously reported that protein lipidation in the form of palmitoylation and farnesylation is critical for the production of A β (amyloid β -peptide), the dimerization of β -secretase and its trafficking into cholesterol-rich microdomains. As statins influence these lipid modifications in addition to their effects on cholesterol biosynthesis, we have investigated the effects of lovastatin and SIMVA (simvastatin) at a range of concentrations chosen to distinguish different cellular effects on A β production and β -secretase structure and its localization in bHEK cells [HEK-293 cells (human embryonic kidney cells) transfected with the Asp-2 gene plus a polyhistidine coding tag] cells. We have compared the changes brought about by statins with those brought about by the palmitoylation inhibitor cerulenin and the farnesyltransferase inhibitor CVFM (Cys-Val-Phe-Met). The statin-mediated reduction in A β production correlated with an inhibition of β -secretase dimerization into its more active form at all concentrations of statin investigated. These effects were

reversed by the administration of mevalonate, showing that these effects were mediated via 3-hydroxy-3-methylglutaryl-CoA-dependent pathways. At low (1 μ M) statin concentrations, reduction in A β production and inhibition of β -secretase dimerization were mediated by inhibition of isoprenoid synthesis. At high (> 10 μ M) concentrations of statins, inhibition of β -secretase palmitoylation occurred, which we demonstrated to be regulated by intracellular cholesterol levels. There was also a concomitant concentration-dependent change in β -secretase subcellular trafficking. Significantly, A β release from cells was markedly higher at 50 μ M SIMVA than at 1 μ M, whereas these concentrations resulted in similar reductions in total A β production, suggesting that low-dose statins may be more beneficial than high doses for the therapeutic treatment of Alzheimer's disease.

Key words: Alzheimer's disease, amyloid β -peptide (A β), cholesterol, dimerization, palmitoylation, β -secretase, statin.

INTRODUCTION

Patients on long-term statin treatment have been shown to have a 70% lower risk of developing AD (Alzheimer's disease) [1,2], and a recent study has shown that ATORVA (atorvastatin) improved the cognitive ability of patients with mild-to-moderate AD [3]. We have previously reported that the production of A β (amyloid β -peptide), whose aggregation and deposition as senile plaques is a pathological hallmark of AD [4], is reduced by LOVA (lovastatin) *in vitro*, which can be reversed by addition of exogenous cholesterol [5]. Inhibition of the final step of cholesterol biosynthesis using BM15.766 reduces A β production [6], suggesting that statins may reduce A β production by lowering cholesterol levels.

However, statins have other cellular effects, the most relevant being an anti-inflammatory effect mediated in part by inhibition of the synthesis of the isoprenoids farnesyl and geranylgeranyl pyrophosphate [7]. There is an inflammatory response in a significant number of AD cases [8]. Non-steroidal anti-inflammatories have been shown to reduce the incidence of AD by 40–50% and improve cognitive ability [9]; however, such treatments can be prohibitively toxic [10]. A β stimulates the release of soluble inflammatory markers from microglia [11], and statins have been shown to reduce microglia activation [7]. Statin-mediated

inhibition of isoprenoid synthesis reduces the release of A β from cells but increases the amount remaining within the cell [12].

Several studies have shown that statin actions on A β release are likely to be mediated by β -secretase. β -Secretase [BACE (β -site amyloid-cleaving enzyme)] is a 140 kDa membrane-associated homodimer [13,14] containing extensive N-glycosylation sites that undergo successive processing and sulfation within the ER (endoplasmic reticulum) and Golgi apparatus [15]. BACE is palmitoylated on Cys⁴⁷⁸, Cys⁴⁸² and Cys⁴⁸⁵, which promotes its recruitment into cholesterol-enriched microdomains (rafts) [16]. Inhibiting the palmitoylation of these residues with the fatty acid synthase inhibitor cerulenin prevents BACE dimerization and changes its intracellular trafficking, resulting in a decrease in A β production [17]. There is also substantial evidence that BACE associates with further proteins, for example BRI3 [18] and nicastrin [19], an integral component of the γ -secretase complex.

BACE is localized primarily to the acidic intracellular compartments such as the endosome and TGN (*trans*-Golgi network) [20]; however, a small yet significant amount also associates with APP (amyloid precursor protein) within cholesterol rafts within the cell and the plasma membrane [21]. BACE does not contain a GPI (glycosylphosphatidylinositol) anchor, an important modification of proteins that associate with cholesterol-rich rafts,

Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; ATORVA, atorvastatin; A β , amyloid β -peptide; BACE, β -site amyloid-cleaving enzyme; buffer R, reducing Laemmli sample buffer; buffer NR, non-reducing Laemmli sample buffer; HEK-293 cells, human embryonic kidney cells; bHEK cells, HEK-293 cells transfected with the Asp-2 gene plus a polyhistidine coding tag; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HRP, horseradish peroxidase; LOVA, lovastatin; MA, mevalonate; PA, palmitic acid; SIMVA, simvastatin; TGN, *trans*-Golgi network.

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but BACE does associate with GPI-coupled proteins that may stabilize BACE within these cholesterol-rich rafts [22].

Although BACE does not contain the necessary isoprenylation consensus sequences, the demonstration by Cole et al. [12] that reduction in isoprenoid synthesis by statins reduces A β production suggests that isoprenoids may still be involved in the production of A β by BACE. We recently demonstrated [17] that inhibiting protein farnesylation using the farnesyltransferase inhibitor CVFM (Cys-Val-Phe-Met) resulted in a concentration-dependent decrease in A β production, inhibition of BACE dimerization and alteration in BACE subcellular localization. This may involve an as-yet unidentified 53 kDa isoprenylated protein that co-purifies with BACE [17].

Statins reduce both cholesterol and isoprenoid synthesis via their inhibition of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase. HMG-CoA reductase catalyses the conversion of HMG-CoA into MA (mevalonate), an intermediate of both isoprenoid and cholesterol synthesis. Concentrations in the range of 1–5 μ M inhibit isoprenoid biosynthesis [23], whereas reduction of cholesterol biosynthesis requires concentrations in the region of 20–40 μ M [5]. We have shown that 40 μ M LOVA inhibits the palmitoylation of the CLR (Cys-Leu-Arg) sequence of the BACE C-terminal tail (residues 482–487) [24]. The marked concentration differences in statin actions may be of relevance for the therapy of AD. We have investigated the concentration-dependence of statin-mediated A β production in an *in vitro* model system, and compared the effects of LOVA and SIMVA (simvastatin) with the effects of cerulenin and CVFM. The results are presented here.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). Statistical analysis was performed using the InStat statistical package (GraphPad, San Diego, CA, U.S.A.) using one-way ANOVA followed by Tukey *post-hoc* comparison test, comparing treated cells with untreated cells (medium only).

Cell culture incubations

We used a cell line of bHEK cells [HEK-293 cells (human embryonic kidney cells) transfected with the *Asp-2* gene plus a poly-histidine coding tag] [5,15] (a gift from GlaxoSmithKline, Harlow, U.K.) cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 2% (v/v) Ultrosor[®] G (Pall BioSeptra, Cergy-Saint-Christophe, France). Ultrosor[®] G is free of cholesterol, PA (palmitic acid) and isoprenoid precursors, requiring cells to rely upon their own *de novo* synthesis for these components. Other workers have studied the processing of BACE in transfected HEK-293 cells [24,27,28]. Cells expressing BACE were selected using Geneticin (250 μ g/ml; Invitrogen, Paisley, U.K.).

The effects of LOVA and SIMVA were studied at 1, 10 and 50 μ M concentrations. Farnesyltransferase activity in the cells was inhibited by the addition of CVFM (0.1 μ M; Alexis, Nottingham, U.K.). Palmitoyltransferase activity was inhibited by the addition of cerulenin (5 μ g/ml). For MA re-incorporation experiments, cells were co-incubated with 50 μ M SIMVA and 0–400 μ M MA. This has been shown to maintain isoprenoid levels while reducing cholesterol synthesis [12,29] and to return A β production back to that observed in untreated cells [30].

ELISA detection of A β

A β production was measured independently in three compartments: released, remaining and total [29]. Released A β consisted

of A β secreted into the culture medium. Remaining A β was obtained by removing medium from the cells, washing once with PBS (Invitrogen) and lysing with RIPA buffer [50 mM Tris/HCl (pH 7.4), 1% (v/v) Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml each of aprotinin, leupeptin and pepstatin, 1 mM sodium vanadate and 1 mM sodium fluoride] [17], diluted 1:10 in PBS (pH 7.4), for 30 min at 4°C. Total A β production was obtained by lysing cells into the culture medium by the addition of 1 $\times$ RIPA buffer (final concentration 0.1 $\times$) to the culture medium and incubating for 30 min at 4°C. Prior to assay, all samples were centrifuged for 10 min at 18 000 *g* using a Hyspin 16K table-top microcentrifuge (Anachem, Luton, U.K.) in order to pellet insoluble material.

A β was quantified using a double-antibody A β ELISA [11] using a combination of rabbit-anti-NTA4 [20,24,25] (2 μ g/ml), monoclonal anti-6E10 (1:2000; IDLabs, Glasgow, U.K.) and anti-(mouse IgG)–HRP (horseradish peroxidase) conjugate (1:500; Amersham Biosciences, Little Chalfont, Bucks., U.K.). The ELISA detected all species of A β monomers, and is linear in the A β concentration range of 1–100 ng/ml [17,26]. The specificity of the ELISA for soluble monomeric A β over oligomeric A β was confirmed by using an A β oligomer ELISA [11] to measure A β oligomers in the synthetic A β _{1–40} samples used to construct the calibration curve for the A β monomer ELISA. The A β oligomer ELISA utilized anti-6E10 as a capture antibody in concert with biotinylated anti-6E10 to detect oligomers within samples. No oligomers were detected in any of the A β standards used (results not shown), demonstrating that the combination of anti-NTA4 and anti-6E10 antibodies was specific for monomeric A β . Samples (50 μ l) were applied in triplicate and incubated for 1.5 h. Colour was developed by the addition of TMB (3,3',5,5'-tetramethylbenzidine) peroxidase substrate (Europa Bioscience, Ely, U.K.). Absorbance at 420 nm was measured using a MultiSkan EX 96-well plate reader (ThermoElectron, Basingstoke, U.K.). Concentrations were calculated from the average of three experiments all performed in triplicate and expressed as A β concentration in ng/ml using standard concentrations of synthetic A β _{1–40}.

Cytotoxicity assay

The cytotoxicity of inhibitors was measured in the bHEK cells using the LDH Cytotoxicity kit (Roche Diagnostics, Lewes, U.K.). Results were calculated from the average of three experiments all performed in triplicate and were expressed as percentage cell death.

BACE and γ -secretase activity assays

BACE and γ -secretase activities were measured using the fluorescence-based β -secretase and γ -secretase assay kits (R&D Systems, Abingdon, U.K.). Results were expressed as specific activities (fluorescence unit $\cdot$ h^{–1} $\cdot$ mg of protein^{–1}), with protein concentrations measured using the Bio-Rad D_c Protein Assay kit (Bio-Rad Laboratories, Hemel Hempstead, U.K.).

Cellular cholesterol assay

Total cellular cholesterol levels were measured using the fluorescence-based Amplex total cholesterol assay (Molecular Probes, Invitrogen). Total cellular cholesterol content was calculated and expressed as μ g of total cholesterol/mg of protein.

Western-blot analysis of BACE

BACE protein was detected in protein lysates using Western blotting as described previously [15] with appropriate

modifications using our own anti-BACE antibody which detects both monomeric (70 kDa) and dimeric (140 kDa) forms of BACE [13,17,20]. As palmitoyl and farnesyl moieties are attached to proteins via reducible thioester bonds, samples were prepared by diluting samples 1:1 in both reducing {Laemmli sample buffer [0.125 M Tris/HCl (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol and 0.004 % Bromophenol Blue]; buffer R} and non-reducing (buffer NR, comprising Laemmli sample buffer without 2-mercaptoethanol) buffers, followed by boiling for 5 min. Protein lysate samples (30 μ l) were electrophoresed on a 4–12 % NuPage Bis-Tris gel (Invitrogen), then transferred on to a PVDF membrane (Waters, Elstree, U.K.). BACE was detected using a combination of a rabbit anti-BACE peptide antibody (1:2000), mouse anti-(rabbit IgG)–HRP conjugate (1:5000) (Amersham Biosciences) and ECL[®] (enhanced chemiluminescence) detection (Amersham Biosciences) using Hyperfilm M (Amersham Biosciences). Molecular masses of visualized proteins were calculated using a calibration plot constructed with standards of known molecular mass and expressed as kDa $\pm$ 9.3 %, calculated as the average error between actual molecular masses of the standards used and their observed molecular masses calculated from the resultant calibration line.

Confocal microscopy

The subcellular localization of BACE was visualized using dual-label confocal microscopy as previously described [5] using a combination of rabbit-anti-BACE (1:200) and a premixed secondary antibody complex consisting of biotinylated mouse-anti-rabbit IgG (1:500; Dako, Ely, U.K.) and streptavidin-linked rhodamine (1:500; Dako). Cholesterol was detected using the cholesterol-binding agent filipin (50 μ g/ml). Fluorescence was observed using a Leica 510 confocal microscope using a $\times$ 40 objective lens, with BACE observed at 543 nm (red fluorescence) and cholesterol observed at 488 nm (green fluorescence).

Incorporation of palmitoyl and farnesyl moieties

Incorporation of palmitoyl using [³H]PA into BACE

In order to assess the effects of LOVA and cholesterol on the incorporation of palmitoyl into BACE, the incorporation of [9,10(*n*)-³H]PA using a method based on that of Benjannet et al. [16] was employed. bHEK cells (5×10^6 cells/flask) were incubated for 16 h with [³H]PA (1 mCi/ml; Amersham Biosciences) in the presence and absence of LOVA. Additional cells were treated with 50 μ M LOVA and 200 μ g/ml cholesterol in CMD (cyclo- β -methyl dextrin) plus [³H]PA for 16 h. BACE was isolated using nickel affinity purification as described previously [13]. Samples prepared in sample buffers R and NR were electrophoresed as above and stained for protein using the PlusOne silver staining kit (Amersham Biosciences). Proteins in which [³H]PA was incorporated were visualized using Amplify fluorographic reagent (Amersham Biosciences), followed by exposure to HyperFilm MP (Amersham Biosciences) for 4 days at -70°C .

Identification of incorporated isoprenyl using [³H]MA

The nature of the isoprenylation of the 53 kDa protein that copurifies with BACE was characterized by assessing the incorporation of [³H]isoprenoid into this protein in the presence and absence of CVFM. Cells were incubated for 16 h with 50 μ M LOVA, after which the medium was replaced with medium supplemented with 50 μ M SIMVA plus [³H]MA (200 μ Ci/ml; Tocris Bioscience, Bristol, U.K.) in the presence or absence of CVFM (0.1 μ M) as appropriate for a further 16 h. Radiolabel incorpo-

ration was assessed as described above, with HyperFilm MP exposed to the gel for 5 weeks at -70°C .

RESULTS

LOVA and SIMVA reduce the production of A β by a mechanism independent of cholesterol

Total A β production was significantly reduced at all concentrations of statin investigated with the exception of 50 μ M LOVA (Figures 1A and 1B), which also gave an increase in cell death (3.9 ± 1.95 % versus 6 ± 2.2 %); however, this was not significant. LOVA (1 and 10 μ M) reduced total A β production by 58 and 67 % respectively ($P < 0.001$), with 50 μ M LOVA resulting in a 11 % decrease in total A β production ($P < 0.05$). A β release was not significantly affected, whereas A β remaining within the cell decreased significantly by 81 and 95 % at 1 and 10 μ M respectively ($P < 0.001$). SIMVA reduced total A β production at all three concentrations used, resulting in a 48 % decrease in total A β production at 50 μ M ($P < 0.01$). In contrast with LOVA, SIMVA significantly increased the release of A β at 10 and 50 μ M, resulting in a 20 and a 66 % increase in A β release respectively ($P < 0.05$ and $P < 0.01$ respectively). A β remaining within the cell decreased significantly at all three concentrations of SIMVA, decreasing by 66, 79 and 85 % respectively ($P < 0.01$). Cerulenin and CVFM resulted in decreases in total A β production of 57 and 54 % respectively (Figure 1C) ($P < 0.001$).

Reduction of total A β production occurred at concentrations (1 μ M) of statins that did not change intracellular cholesterol levels as well as at concentrations at which intracellular cholesterol was significantly reduced (Table 1), suggesting that at low (1 μ M) concentrations, statins were exerting their effects on A β production via cholesterol-independent pathways. At higher (> 10 μ M) concentrations, the cholesterol-dependent pathways may become more important, as intracellular cholesterol was significantly reduced at this concentration for both statins. Cholesterol reduction was concomitant with a statin concentration-dependent change in BACE trafficking (Figure 2). In untreated cells, BACE was co-localized with cholesterol within the Golgi apparatus and associated vesicles. Staining was punctate, with discrete areas of intense staining concurrent with cholesterol raft localization. There was very little staining associated with other compartments within the cell. With increasing LOVA concentration, the punctate staining associated with cholesterol raft localization was replaced with diffuse staining throughout the membrane. Staining associated with BACE became more pronounced in intracellular locations with increasing LOVA concentration. Co-localization of BACE with non-raft associated cholesterol increased with increasing LOVA concentration, resulting in an increase in orange/yellow staining in merged immunofluorescence images (Figures 2B and 2C). Staining associated with cholesterol decreased with increasing LOVA concentration, which coincided with the measured decrease in total cellular cholesterol (Table 1). This decrease in green fluorescence associated with cholesterol staining resulted in the staining associated with BACE within membranes and intracellular regions becoming more orange in appearance at 50 μ M LOVA (Figure 2D). Treatment of the cells with SIMVA, cerulenin or CVFM (results not shown) produced similar effects.

There was no correlation between A β production and the *in vitro* activities against synthetic substrates of either BACE or γ -secretase released from cells with detergent after incubation with statins (Table 2), suggesting that statins are not competitive inhibitors of BACE. There was also no correlation between A β production and cell death, as there was no significant increase

Figure 1 LOVA and SIMVA reduce total A β production

Cells were incubated with 0, 1, 10 and 50 μ M statin, 5 μ g/ml cerulenin or 0.1 μ M CVFM for 16 h prior to assay. A β was measured in the removed medium (Released), lysed cells with medium (Total) and lysed cells (Remaining) using an A β ELISA assay and expressed as ng/ml $\pm$ S.D., with statistical analysis of untreated versus statin-treated cells performed using one-way ANOVA with Tukey *post-hoc* comparisons. Results were calculated as the average of three independent experiments, with each experiment assayed in triplicate. **(A)** Effect of LOVA on A β release (top panel), A β remaining within the cell (middle panel) and total A β production (bottom panel). * P < 0.05; *** P < 0.001. **(B)** Effect of SIMVA on A β release (top panel), A β remaining within the cell (middle panel) and total A β production (bottom panel). * P < 0.05; ** P < 0.01. **(C)** Effect of CVFM and cerulenin on A β release, A β remaining within the cell and total A β production. *** P < 0.001. Black bars, untreated cells; white bars, 0.1 μ M CVFM; hashed bars, cerulenin.

in cell death at any of the concentrations of statin investigated (results not shown).

LOVA and SIMVA inhibit the dimerization of BACE

As statins are also able to inhibit both palmitoyl transfer to BACE [24] and farnesyl biosynthesis, their effects on BACE dimerization were assessed using Western-blot analysis (Figure 3). In cells grown in Ultrosor[®] G medium alone, BACE consisted of two proteins of 145.5 and 73.7 kDa, corresponding to the predicted molecular masses of the dimeric and monomeric forms of glycosylated and lipidated BACE as reported previously [13,14,17]. Treatment of the cells with either LOVA or SIMVA resulted in a single monomeric BACE protein of 73.7 kDa (samples pre-

pared with reducing agent, buffer R) or 65.5 kDa (samples prepared in the absence of reducing agent, buffer NR) at 1, 10 and 50 μ M. Treatment of cells with cerulenin (73.7 and 67.5 kDa for buffers R and NR respectively) and CVFM (73.7 and 69.3 kDa) gave similar results. The differences in mobilities produced by reducing agent in the sample buffer may be caused by release of covalent modifications linked via thioester bonds or the reduction of internal disulfide bonds.

MA addition reverses statin-induced reduction of A β levels and promotes the dimerization of BACE

The inhibition of A β production by 50 μ M SIMVA was overcome by the addition of MA in a dose-dependent manner (Figure 4),

Table 1 Effect of LOVA and SIMVA on total cellular cholesterol concentration

Total cholesterol concentration, measured as free and esterified cholesterol, was calculated as the average of three experiments each performed in triplicates expressed as μg of cholesterol/mg of protein. ns, not significant; * $P < 0.05$ and ** $P < 0.01$.

	Cholesterol concentration
Untreated	785.7 ± 56.2
Lovastatin	
1 μM	$694.9 \pm 40.0^{\text{ns}}$
10 μM	$602.2 \pm 23.5^*$
50 μM	$682.4 \pm 31.8^*$
Simvastatin	
1 μM	$615.4 \pm 75.9^{\text{ns}}$
10 μM	$590.2 \pm 32.7^{**}$
50 μM	$569.6 \pm 24.7^{**}$

with almost 100 % A β production restored at 400 μM MA. This was concomitant with the reappearance of the dimeric form of BACE (Figure 4). The addition of MA did not return total cellular cholesterol levels back to those observed in untreated cells, nor did they differ from those observed in cells incubated with 50 μM SIMVA alone (Table 3). Therefore the inhibition of A β production and BACE dimerization by statins was mediated via inhibition of HMG-CoA reductase-dependent isoprenoid synthesis.

BACE palmitoylation is regulated by intracellular cholesterol levels

Our previous studies have shown that palmitoylation is not dependent on prior protein farnesylation [17], and it is possible that palmitoylation is mediated by alterations in cellular cholesterol levels. In cells grown in Ultrosor[®] G medium alone, [³H]PA was incorporated into both dimeric and monomeric forms of BACE (Figure 5A), resulting in labelled proteins of 141 and 75 kDa respectively. Boiling in the presence of the reducing agent 2-mercaptoethanol removed all trace of radiolabelled protein, showing that [³H]PA was attached via a reducible thioester bond.

Incubation of cells with LOVA reduced the incorporation of [³H]PA in a dose-dependent manner. [³H]PA incorporation occurred into a single protein of 73 kDa at 1 μM LOVA (Figure 5A). As BACE dimerization was inhibited at this concentration (Figure 3), this suggests that BACE palmitoylation is not inhibited at this concentration and does not require prior BACE dimerization. [³H]PA incorporation was reduced in monomeric BACE of 73 kDa by 10 μM LOVA, whereas [³H]PA incorporation was totally abolished by 50 μM LOVA. The addition of 200 $\mu\text{g}/\text{ml}$ cholesterol to cells treated with 50 μM LOVA resulted in the incorporation of [³H]PA into two proteins of 141 and 75 kDa (Figure 5A), which corresponded to the appearance of both dimeric and monomeric BACEs on Western blot (Figure 5B). The farnesyltransferase inhibitor CVFM had no effect on palmitoylation of monomeric BACE, further supporting the hypothesis that statins inhibit BACE palmitoylation via their influence on intracellular cholesterol levels and do not require prior BACE dimerization. Cerulenin inhibited the palmitoylation of both forms of BACE.

Statins inhibit farnesylation of a 53 kDa protein

In order to determine whether the isoprenylation present on the 53 kDa protein is farnesyl or geranylgeranyl, the incorporation of [³H]MA in the presence and absence of CVFM was investigated. As previously observed, there was significant isoprenyl incorporation into many bHEK proteins (Figure 6). Boiling protein samples in buffer R removed all [³H]isoprenoid label, showing

Figure 2 LOVA changes subcellular trafficking of BACE

Cells were incubated with 0, 1, 10 and 50 μM LOVA, 5 $\mu\text{g}/\text{ml}$ cerulenin or 0.1 μM CVFM, after which BACE expression was detected using confocal microscopy using a combination of anti-BACE (1:200), biotinylated mouse anti-rabbit IgG (1:500) and streptavidin-linked rhodamine (1:500). Cholesterol localization was determined using the cholesterol marker filipin (50 $\mu\text{g}/\text{ml}$). Fluorescent staining was observed with a Leica 510 confocal microscope using an excitation wavelength of 543 nm for BACE (red fluorescence) and an excitation wavelength of 488 nm for cholesterol (green fluorescence). BACE and cholesterol co-localization was observed as orange/yellow fluorescence. In untreated cells (A), BACE was co-localized with cholesterol within the Golgi apparatus and associated vesicles (arrows). Staining was punctate, corresponding to cholesterol raft localization. There was no staining within other intracellular areas of the cell (*). With increasing concentrations of LOVA (B, C), BACE localization became much more general throughout the cell (*), with staining within the membrane becoming much more diffuse (arrows). This resulted in an increase in co-localization with cholesterol (orange/yellow staining). At 50 μM LOVA, BACE localization became much more diffuse throughout the membrane, with staining present in intracellular areas (*). However, due to the decrease in staining associated with cholesterol arising from decreased cellular cholesterol, this staining was predominantly orange in appearance, with discrete areas of yellow staining in a very small number of areas (arrows) (D). These changes were in accord with changes in BACE localization elicited by cerulenin and CVFM (results not shown). (A) Untreated cells; (B) 1 μM LOVA; (C) 10 μM LOVA; (D) 50 μM LOVA. Scale bar, 10 μm .

that the prenylation modifications were attached to proteins via thioester bonds. The isoprenylation of the 53 kDa protein was inhibited by CVFM, showing that this protein is farnesylated. The farnesylated 53 kDa protein co-purified with BACE on a nickel

Table 2 Effect of statins, cerulenin and CVFM on BACE and γ -secretase activity

Specific activity (SA), calculated from three independent experiments in which each experiment was performed in triplicate, is expressed as fluorescent units $\cdot$ h⁻¹ $\cdot$ (mg of protein)⁻¹. ns, not significant; ***P* < 0.01 and ****P* < 0.001.

	BACE activity (SA $\pm$ S.D.)	γ -Secretase activity (SA $\pm$ S.D.)
Untreated	34 288 $\pm$ 1824	759.8 $\pm$ 39.6
Lovastatin		
1 μ M	22 707 $\pm$ 1143***	729.6 $\pm$ 8 ^{ns}
10 μ M	47 685 $\pm$ 1571***	925.5 $\pm$ 26.8**
50 μ M	27 508 $\pm$ 1394***	812.4 $\pm$ 10.1 ^{ns}
Simvastatin		
1 μ M	30 186 $\pm$ 171**	1192.1 $\pm$ 54.5***
10 μ M	29 702 $\pm$ 1437**	629.2 $\pm$ 2.58**
50 μ M	40 326 $\pm$ 1632***	1231.9 $\pm$ 35.1***
Cerulenin		
5 μ g/ml	30 329 $\pm$ 1901 ^{ns}	1377.8 $\pm$ 85.6***
CVFM		
0.1 μ M	31 692 $\pm$ 678 ^{ns}	738.6 $\pm$ 10.8 ^{ns}

Figure 3 LOVA and SIMVA inhibit BACE dimerization

Cells were incubated as per Figure 1, and BACE was detected using Western-blot analysis with the following antibody combination: anti-BACE (1:2000), overnight at 4°C; mouse anti-(rabbit IgG)-HRP conjugate (1:5000), 1 h at room temperature. Molecular masses of detected proteins were calculated using a calibration line constructed using standards of known molecular mass and expressed as kDa. Samples were loaded in couplets, the first lane of each comprising a sample boiled for 5 min in buffer R, and the second lane of each couplet boiled for 5 min in buffer NR. Lanes 1 and 2, untreated cells; lanes 3 and 4, 1 μ M LOVA; lanes 5 and 6, 10 μ M LOVA; lanes 7 and 8, 50 μ M LOVA; lanes 9 and 10, 1 μ M SIMVA; lanes 11 and 12, 10 μ M SIMVA; lanes 13 and 14, 50 μ M SIMVA; lanes 15 and 16, 5 μ g/ml cerulenin; lanes 17 and 18, 0.1 μ M CVFM.

affinity-purification column, suggesting that the two proteins may be part of the same complex.

DISCUSSION

As A β is implicated by the amyloid hypothesis as a causative agent in AD, BACE and γ -secretase offer potential targets for drugs for the disease. Recent evidence suggesting that statin use reduces the risk of AD makes it important to fully elucidate the mechanisms by which statins influence A β production. The present study demonstrates that statins inhibit the dimerization of BACE via HMG-CoA reductase-dependent pathways, involving both isoprenoid synthesis and intracellular cholesterol levels, which in turn regulates the palmitoylation of BACE. These results provide further evidence that physiologically active BACE exists as a complex of proteins, whose assembly is reliant upon protein lipidation reactions.

There is a dose-dependent effect of statins on cellular cholesterol and isoprenoid levels

The differences in the concentrations of statins required to inhibit both isoprenoid and cholesterol biosyntheses [20,25,30], in which we observed significant reduction in cellular cholesterol levels at LOVA and SIMVA concentrations greater than 10 μ M (Table 1),

Figure 4 MA reverses the effects of statins on A β production and BACE dimerization

Cells were incubated with 50 μ M SIMVA and 0–400 μ M MA for 16 h prior to ELISA and Western-blot analysis. Total A β production was measured using an A β ELISA assay and expressed as ng/ml $\pm$ S.D., with statistical analysis of untreated versus MA-treated cells performed using one-way ANOVA with Tukey *post-hoc* comparisons. Results were calculated as the average of three independent experiments, with each experiment assayed in triplicate. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant. BACE was detected using Western-blot analysis as per Figure 3, using samples prepared by boiling for 5 min in non-reducing buffer only. Molecular masses were calculated using a calibration line produced from standards of known molecular mass and expressed as kDa. Upper arrow, 140 kDa; lower arrow, 70 kDa.

Table 3 Effect of co-incubation of 50 μ M SIMVA and 0–400 μ M MA on total cellular cholesterol levels

Total cholesterol concentration, measured as free and esterified cholesterol, was calculated as the average of triplicates and expressed as percentage of total cellular cholesterol concentration in untreated cells, which was taken as 100%. In all incubations, cells were co-incubated with 50 μ M SIMVA and 0–400 μ M MA, with the exception of untreated cells, which were incubated in Ultrosor® G medium alone.

MA concentration (μ M)	Cholesterol concentration (% of untreated $\pm$ S.D.)	Versus untreated*	Versus 50 μ M SIMVA†
Untreated	100 $\pm$ 5.63	–	–
0	72.5 $\pm$ 2.97	<i>P</i> < 0.001	–
50	55.6 $\pm$ 2.84	<i>P</i> < 0.001	ns
100	75.6 $\pm$ 2.00	<i>P</i> < 0.001	ns
250	53.5 $\pm$ 1.85	<i>P</i> < 0.001	ns
400	78.2 $\pm$ 6.82	<i>P</i> < 0.001	ns

* Cellular cholesterol levels in cells co-incubated with 50 μ M SIMVA and increasing concentrations of MA were compared with cholesterol levels in cells grown in Ultrosor® G medium only to determine whether increasing concentrations of MA were able to reverse the reduction of total cellular cholesterol levels by 50 μ M back to those levels observed in untreated cells.

† Cellular cholesterol levels in cells pretreated with 50 μ M SIMVA and increasing concentrations of MA were compared with cellular cholesterol levels in cells pretreated with 50 μ M SIMVA alone to determine whether total cellular cholesterol levels differed at any of the concentrations of MA investigated. ns, not significant.

may reflect the differences in the way cellular levels of isoprenoids and cholesterol are maintained. There are two pools of cholesterol, free cholesterol and esterified cholesterol. Esterified

Figure 5 BACE palmitoylation is mediated by cholesterol

Cells were incubated for 16 h in the presence of [3 H]PA (1 mCi/ml) in normal medium or medium supplemented with 1, 10 and 50 μ M LOVA, 5 μ g/ml cerulenin or 0.1 μ M CVFM, or in medium supplemented with 50 μ M LOVA and 200 μ g/ml cholesterol. (A) Effect of statins and cholesterol on the incorporation of [3 H]PA into BACE. After incubation, cells were lysed and then BACE was purified using nickel affinity purification and subjected to SDS/PAGE. Radiolabel incorporation was visualized using fluorography, with gel exposure at -70°C for 4 days. Molecular masses of detected proteins were expressed as kDa. Each sample was prepared in either buffer NR (odd-numbered lanes) or buffer R (even-numbered lanes), which were loaded in adjacent lanes, in order to see if [3 H]PA label was attached via thioester linkages. The exceptions to this were cerulenin- and CVFM-treated samples, which were prepared in buffer NR only. Lanes 1 and 2, untreated cells; lanes 3 and 4, 1 μ M LOVA; lanes 5 and 6, 10 μ M LOVA; lanes 7 and 8, 50 μ M LOVA; lanes 9 and 10, 50 μ M LOVA + 200 μ g/ml cholesterol in CMD; lane 11, 5 μ g/ml cerulenin; lane 12, 0.1 μ M CVFM. (B) Effect of cholesterol on BACE dimerization. BACE was detected using Western-blot analysis as described above, with samples prepared in buffer NR. Molecular masses of detected proteins were calculated and expressed as kDa. Lane 1, untreated cells; lane 2, 50 μ M LOVA; lane 3, 50 μ M LOVA + 200 μ g/ml cholesterol.

Figure 6 A 53 kDa protein that co-purifies with BACE is farnesylated

Cells were incubated for 16 h with 50 μ M LOVA, followed by further overnight incubation with 50 μ M LOVA plus [3 H]MA (200 μ Ci/ml) in the presence and absence of CVFM (0.1 μ M). Cells were lysed and BACE was purified using nickel affinity purification. Samples were prepared in buffer NR only in order to ensure that any thioester-linked radiolabelled isoprenyl moieties were not removed. Samples were electrophoresed and radiolabel incorporation was determined as described in Figure 5, with gel exposure at -70°C for 5 weeks. Molecular masses of detected proteins were calculated and expressed as kDa. This Figure is a typical result constructed from three experiments and is representative of results obtained. Lanes 1 and 2, all radiolabelled proteins from cells incubated in the absence of CVFM prepared in non-reducing and reducing buffers respectively; lane 3, purified BACE sample from cells incubated in the absence of CVFM; lane 4, purified BACE sample from cells incubated in the presence of 0.1 μ M CVFM.

cholesterol acts as a metabolic reserve of cholesterol that can be converted back into cholesterol by cholesterol esterase in times of low cellular cholesterol levels. This reduces the reliance of the cell on *de novo* cholesterol synthesis in order to maintain cellular cholesterol levels. In contrast, isoprenoids have no such metabolic reserve, and rely solely on *de novo* synthesis in order to maintain cellular isoprenoid levels. This would make cellular isoprenoid levels much more sensitive to HMG-CoA reductase inhibition than cellular cholesterol levels, also resulting in preferential isoprenoid synthesis over cholesterol synthesis in the presence of MA, resulting in isoprenoid synthesis utilizing most of the MA passing down this shared metabolic pathway. This is in accord with our results that show no effect of MA on cellular cholesterol levels after 50 μ M LOVA incubation and is also in agreement with studies by other laboratories that have shown that MA re-incorporation after statin inhibition of HMG-

CoA reductase preferentially increases isoprenoid synthesis over cholesterol synthesis [12,29].

Statins inhibit the dimerization of BACE by inhibiting isoprenoid synthesis

Our study is the first demonstration that BACE dimerization can be inhibited by statins via HMG-CoA reductase-dependent isoprenoid biosynthesis. Blocking the dimerization of BACE is an attractive therapeutic target, as the dimer is 30-fold more active at cleaving APP than the monomer [14] and so inhibiting BACE dimerization would reduce A β production markedly.

BACE palmitoylation is mediated via cholesterol

LOVA treatment yielded dose-dependent concomitant changes in BACE subcellular trafficking and inhibition of palmitoylation, complete at 50 μ M LOVA, which were reversed by the addition of exogenous cholesterol (Figure 5), showing that both are connected to the cholesterol gradient in much the same way as the maturation of the glycosylation of BACE [31]. The palmitoylation of BACE does not require prior dimerization, as monomeric BACE was palmitoylated in the presence of both 1 μ M LOVA and 0.1 μ M CVFM. It is also clear that palmitoylation is required, although not sufficient in itself, for dimerization to occur, as inhibiting palmitoylation using cerulenin inhibits the dimerization of BACE (Figure 3) [17]. It is therefore likely that the cholesterol gradient may play a part in the dimerization process, as re-introducing cholesterol after 50 μ M LOVA treatment results in the dimerization of BACE and re-incorporation of [3 H]PA into both dimeric and monomeric forms of BACE. Palmitoylation is usually required by proteins to stabilize their association with membranes [32], an association that BACE requires for dimerization to occur [14]. Therefore, in the temporal sequence of processing of BACE, palmitoylation occurs prior to BACE dimerization and prosequence cleavage, which are driven by the cholesterol gradient. Inhibiting cellular cholesterol biosynthesis by LOVA lowers the intracellular cholesterol gradient [33,34], therefore reducing the cholesterol-driven palmitoylation and dimerization of BACE.

BACE undergoes palmitoylation and associates with a farnesylated 53 kDa protein in a sequential manner

It is common for protein palmitoylation to require prior farnesylation [32]; however, BACE is not farnesylated [17]. There is mounting evidence that BACE exists as a complex within cholesterol-rich rafts [22], and although many associations with other proteins may be transitory in nature, some associations may be permanent. The association of BACE with a 53 kDa farnesylated protein [17] would indirectly provide BACE with a farnesyl group necessary to stabilize BACE within its lipid raft localization. We propose that BACE undergoes a timed sequence series of maturation steps prior to entry into the cholesterol-rich rafts, as summarized in Figure 7. After production within the ER and during its cholesterol gradient-driven processing through the TGN, BACE undergoes palmitoylation, which is followed by dimerization and association with a 53 kDa farnesylated protein. This in turn stabilizes BACE within the membrane, enabling it to undergo prosequence cleavage and subsequent recruitment into cholesterol-rich microdomains.

The model suggests several potential therapeutic targets that could be exploited in order to disrupt the assembly of the BACE complex. Inhibition of any of these steps would prevent the dimerization of BACE and its association with cholesterol-rich rafts, all of which we have demonstrated experimentally. Such

Figure 7 Schematic representation of the palmitoylation of BACE, its association with farnesylated 53 kDa protein and its stabilization into its dimeric physiological form

Upon synthesis, BACE associates with the ER membrane (1) where it undergoes cholesterol-driven palmitoylation (2), stabilizing its association with the membrane. BACE then dimerizes (3) upon association with farnesylated 53 kDa protein (4), stabilizing its dimeric structure within the membrane. BACE's prosequence is then cleaved (5), forming the physiological BACE complex, which then enters the cholesterol-rich rafts. Palmitoylation of BACE's C-terminal tail (closed circles) and association with the farnesylated 53 kDa protein (open circles) provide the dual lipidation common in lipid-raft-associated proteins.

a strategy would channel APP processing via the non-amyloidogenic α -secretase pathway. Transgenic BACE knockout animal models show no pathological or behavioural abnormalities [35], which suggests that BACE is not necessary for survival. In contrast, BACE activity and expression increase as a consequence of natural aging [36], and studies have shown a small but significant increase in BACE activity in patients with AD compared with non-disease controls [37]. As increased $A\beta$ load is associated with an increase in AD pathology [4], targeting the assembly of the BACE complex may be an effective therapeutic strategy.

SIMVA increases the release of $A\beta$ from bHEK cells

The decrease in $A\beta$ intracellular accumulation and production by low concentrations ($< 10 \mu\text{M}$) of statins was not unexpected. Previous studies have reported reductions in total $A\beta$ production and accumulation of up to 50% in HEK-293 cells by concentrations of LOVA and SIMVA below $10 \mu\text{M}$ [5,38], and up to a 40% reduction in intracellular accumulation of $A\beta$ by $4 \mu\text{M}$ LOVA and SIMVA in isolated rat hippocampal neurons [29,39]. In contrast, Cole et al. [12] have recently demonstrated that $10 \mu\text{M}$ LOVA increases the intracellular accumulation of $A\beta_{1-40}$ by 400% in both HEK-293 and SH-SY5Y cells. It is unclear as to why this is the case, but it may represent methodological differences between studies.

However, we are the first to demonstrate an increase in $A\beta$ release in response to incubation with SIMVA. SIMVA at 10 and $50 \mu\text{M}$ decreased cellular cholesterol levels (Table 1) and increased $A\beta$ release from bHEK (Figure 1). This is in contrast with previous studies which have reported a 50–90% reduction in $A\beta$ release from cells treated with $10 \mu\text{M}$ SIMVA [3,29,38,39]; however, it is an effect that we have previously observed [26]. This leakage of $A\beta$ that we observe most likely arises from the reduction in plasma membrane cholesterol [33,34], which would make it more permeable to small molecules such as $A\beta$ and thus result in the statin concentration-dependent increase in $A\beta$ that we observe.

LOVA and SIMVA have different effects on $A\beta$ production

It is clear from our data that LOVA and SIMVA, although they both have the same effect on $A\beta$ production, differ in the degree to which they have their effects. The percentage reductions in remaining and total $A\beta$ was less for SIMVA than for LOVA, suggesting that SIMVA is less effective at reducing $A\beta$ production than LOVA. Such a difference is not unexpected, as different statins exhibit different abilities in inhibiting microglia activation [40]. Such differences between statins are not limited to *in vitro* studies. Wolozin et al. [2] reported that the prevalence of AD in patients receiving LOVA therapy was significantly reduced compared with the total patient population within their study, whereas the prevalence of AD in patients receiving SIMVA did not differ from the total patient population.

The reasons for these differences are unclear. LOVA and SIMVA, which differ structurally by an additional methyl group, are transported into the cell via passive diffusion across the membrane, and have correspondingly similar IC_{50} values for the inhibition of HMG-CoA reductase [34]. Additionally, both statins are metabolized by CYP3A4 (where CYP is cytochrome P450) [34].

Recently, it has become possible to obtain SIMVA over-the-counter without the need for a prescription. Taking into consideration both our data showing a greater effect of LOVA on $A\beta$ production compared with SIMVA and the reduction in the prevalence of AD by LOVA as reported by Wolozin et al. [2], it is likely that this change in prescription status for SIMVA will have little or no effect on the incidence of AD in the general population and that the use of LOVA would be more beneficial.

Low-dose statin therapy may be beneficial for the treatment of AD

Since the original retrospective reports showing a reduction in the incidence of AD in patients taking long-term statins, there has been much discussion regarding their use as an anti-AD treatment. Initial promising results have not been replicated in large-scale studies, with little effect seen on cognitive decline. A recent study has demonstrated a potentially beneficial effect of ATORVA on the cognitive decline in patients with mild AD; however, the difference observed was small [3]. The 80 mg/day dosage of ATORVA used in these clinical studies is the maximal recommended dose for patients [29] and therefore represents statin dosage at the highest level. We have previously shown that statins at concentrations that reduce cholesterol production ($20 \mu\text{M}$) reduce the total production of $A\beta$ in bHEK cells, yet increase its release from the cell [26]. Our present results confirm this finding, while showing that lower doses may be just as effective at reducing total $A\beta$ production. At $1 \mu\text{M}$ SIMVA, we observed a 42% decrease in total $A\beta$ produced, which differed from $50 \mu\text{M}$ by a mere 5%, suggesting that low doses may be just as effective as high doses.

This benefit may not be solely limited to the low dose required, however. The amount of $A\beta$ released from cells increased by 66% at $50 \mu\text{M}$ SIMVA, compared with a 20% increase at $1 \mu\text{M}$. The observed increase in release has ramifications for the use of statins in AD treatment. If this were to occur in the *in vivo* human brain, this increase in $A\beta$ release at high statin concentrations may have detrimental consequences. An increase in $A\beta$ production under these conditions may accelerate the oligomerization of $A\beta$ into toxic aggregates, which may therefore be masking the beneficial effects of statins in previous clinical studies. The bioavailability of both LOVA and SIMVA is below 5%, while the bioavailability of ATORVA is 12% [34]. With the standard dose of ATORVA (40 mg/day), this would result in a plasma concentration

of approx. 1–5 nM. Statins have a very high affinity for HMG-CoA reductase, in the region of 1.2–55 nM [34], which places the maximal ATORVA concentration within the upper end of this range. Therefore use of low-dose statins may demonstrate a more beneficial effect in such trials.

The concentrations used in our study are in line with others who have used statins in their studies [3,5,12,23,29,30,38,39]. However, the differences in concentrations required to reduce cholesterol synthesis *in vivo* and *in vitro* may reflect differences in statin metabolism. *In vitro*, alterations in plasma cholesterol levels require a number of weeks of administration of statins. In contrast, the decreases in cellular cholesterol levels observed in *in vitro* studies occur within a very short time frame. Both SIMVA and LOVA are prodrugs, administered in their lactone form, which undergo subsequent reversible conversion within the body into their active open acid forms [34,41]. It is probable that these interconversions, although they may occur *in vitro*, do not occur to a significant degree in comparison, and therefore much higher concentrations of statins are required in order to achieve physiological effects. It is therefore probable that the effective concentration of statin open acid forms used *in vitro* may well be within the low nanomolar range. It is also common to administer high doses of statin in *in vivo* models in order to obtain a rapid decrease in cellular cholesterol levels [29]. Irrespective of these considerations however, the concentrations used in the present study were chosen to allow the investigation of the cholesterol and isoprenoid effects of statins, and we have demonstrated that each of these lipid effects mediate the production of A β via BACE.

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