Statins Cause Intracellular Accumulation of Amyloid Precursor Protein, β-Secretase-cleaved Fragments, and Amyloid β-Peptide via an Isoprenoid-dependent Mechanism*

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The use of statins, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors that block the synthesis of mevalonate (and downstream products such as cholesterol and nonsterol isoprenoids), as a therapy for Alzheimer disease is currently the subject of intense debate. It has been reported that statins reduce the risk of developing the disorder, and a link between cholesterol and Alzheimer disease pathophysiology has been proposed. Moreover, experimental studies focusing on the cholesterol-dependent effects of statins have demonstrated a close association between cellular cholesterol levels and amyloid production. However, evidence suggests that statins are pleiotropic, and the potential cholesterol-independent effects of statins on amyloid precursor protein (APP) metabolism and amyloid β-peptide (Aβ) genesis are unknown. In this study, we developed a novel in vitro system that enabled the discrete analysis of cholesterol-dependent and -independent (i.e. isoprenoid-dependent) statin effects on APP cleavage and Aβ formation. Given the recent interest in the role that intracellular Aβ may play in Alzheimer disease, we analyzed statin effects on both secreted and cell-associated Aβ. As reported previously, low cellular cholesterol levels favored the α-secretase pathway and decreased Aβ secretion presumably within the endocytic pathway. In contrast, low isoprenoid levels resulted in the accumulation of APP, amyloidogenic fragments, and Aβ likely within biosynthetic compartments. Importantly, low cholesterol and low isoprenoid levels appeared to have completely independent effects on APP metabolism and Aβ formation. Although the implications of these effects for Alzheimer disease pathophysiology have yet to be investigated, to our knowledge, these results provide the first evidence that isoprenylation is involved in determining levels of intracellular Aβ.

A growing body of evidence suggests that the amyloid β-peptide (Aβ) plays a critical and early role in Alzheimer disease (AD) pathogenesis. AD is characterized by cerebral amyloid plaques, which are extracellular deposits of Aβ (1, 2). Overproduction of the 42-amino acid form of Aβ (Aβ42) is associated with early onset familial AD, and Aβ42 appears toxic to neurons in vitro and in vivo (reviewed in Refs. 3 and 4). Moreover, recent reports suggest that, in addition to extracellular Aβ, the accumulation of intracellular Aβ may be involved in AD (5–15). Thus, much research has been devoted to understanding the role of Aβ in AD and to developing therapeutic strategies for reducing Aβ levels or toxicity.

Aβ is cleaved from amyloid precursor protein (APP) by two proteases, the β- and γ-secretases (reviewed in Refs. 4, 16, and 17). Initially, β-secretase (also known as BACE1) cuts APP at the N terminus of the Aβ domain to produce the membrane-bound APP C-terminal fragment (CTF) C99 and the secreted APP ectodomain APPsβ. C99 is the substrate of γ-secretase, which cleaves to generate the C terminus of Aβ. γ-Secretase cleavage is heterogeneous and produces Aβ peptides of different lengths (~38–43 amino acids). An alternative non-amyloidogenic pathway also occurs in which a third protease, α-secretase, cuts APP within the Aβ domain, thus precluding Aβ formation. α-Secretase cleavage produces the membrane-bound APP CTF CS8 and the secreted APP ectodomain APPsα. Like C99, CS8 is a substrate of γ-secretase, which cleaves to generate the non-amyloidogenic p3 fragment. Previous studies indicate that the α- and β-secretase pathways may compete for APP substrate under certain conditions such that increased activity of one pathway leads to decreased APP processing in the other (18, 19). Although Aβ42 is implicated as a causative factor for early onset familial AD, the link between Aβ and the pathogenesis of late onset sporadic AD is less firmly established. Factors that increase the risk of late onset AD have been identified, but their relationship to Aβ is unclear. Of particular interest are genetic and environmental factors that affect cholesterol metabolism and that associate with AD. For example, the apoE4 isoform is a risk factor for AD and is linked to increased serum cholesterol levels (20–22). Atherosclerosis and stroke, conditions that share hypercholesterolemia as a risk factor, appear to be associated with AD as well (23–26). Indeed, epidemiological studies indicate that...
high serum cholesterol levels increase the risk of AD, and it has been proposed that the homeostatic regulation of cholesterol metabolism may be altered in AD (27). In contrast, recent reports show a significant reduction in AD risk for patients treated with statins, a group of cholesterol-lowering drugs (28, 29). Taken together, these studies suggest that reduction of cholesterol levels may inhibit AD pathogenesis.

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis (reviewed in Refs. 30 and 31). HMG-CoA reductase converts HMG-CoA into mevalonate (see Fig. 1A), and inhibition of HMG-CoA reductase reduces the synthesis of all mevalonate pathway products. Indeed, mevalonate is a precursor of not only cholesterol, but also of many nonsterolid isoprenoids, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (see Fig. 1A). Isofrenylation is a functionally important post-translational modification of a variety of proteins, including small GTPases (e.g., Ras, Rab, and Rho), and plays a crucial role in protein trafficking and signaling, cytoskeletal structure, cell motility, and membrane transport (32, 33). Thus, it has been postulated that statins may have significant cholesterol-independent effects that result from inhibition of the isoprenoid pathway.

Recent work has suggested that the AD risk reduction associated with statin treatment may be the result of decreased amyloidogenic APP processing caused by low cellular cholesterol levels. Treatment with statins or depletion of cholesterol with MβCD appears to increase α-secretase cleavage of APP in cells, whereas β-secretase cleavage and secreted Aβ levels are decreased (34–39). Conversely, cholesterol enrichment leads to elevated amyloidogenic processing of APP (40–42). Although these results suggest that cellular cholesterol levels modulate APP processing, other reports indicate that cholesterol esters (rather than free cholesterol) affect the activities of the secretases such that low cholesterol ester levels decrease Aβ formation (43). In addition, the subcellular distribution of cholesterol may also influence APP cleavage because mutations and pharmacological inhibitors of the Niemann-Pick complex cholesterol transport pathway alter the localization of presenilin/γ-secretase and lead to greater Aβ production (44–47). Thus, the form, level, and distribution of cholesterol in cells may modulate APP processing in a complex fashion.

Statins inhibit both cholesterol and isoprenoid synthesis, and therefore, it was important to determine their individual effects on APP processing because previous studies had not done so. To this end, in this work we investigated the cholesterol-dependent versus isoprenoid-dependent effects of statin treatment on APP cleavage and Aβ formation in cells. By independently manipulating total cellular cholesterol and isoprenoid levels during exposure to statins, we found that cholesterol and isoprenoids have differential effects on the secretion and intracellular accumulation of FL-APP, APP fragments, and Aβ. As expected from previous studies, low cellular cholesterol levels favor the α-secretase pathway and decrease Aβ secretion. However, we also obtained the novel result that low isoprenoid levels cause cell-associated accumulation of FL-APP, APP fragments, and intracellular Aβ, and these effects were reversed by supplementation with a low concentration of mevalonate or GGPP.

Interestingly, low isoprenoid and cholesterol levels appear to act through unrelated mechanisms. In particular, the secreted and intracellular pools of Aβ behave independently of one another. Understanding both the cholesterol-dependent and isoprenoid-dependent statin effects on APP processing may shed light on the mechanisms involved in statin-mediated AD risk reduction and the accumulation of intracellular Aβ, which may play a role in AD pathophysiology.
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Immunoblot Analysis—Following treatment, the conditioned media were harvested, and cells were scraped into lysis buffer (20 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, and 0.01% sodium azide) with protease inhibitor cocktail, and the protein concentration was measured with using the BCA protein assay kit. Equal amounts of protein (20 μg/lane for detection of APP CTFs and 10 μg/lane for all other proteins) were boiled under reducing conditions, loaded onto either NuPAGE 4–12% BisTris gels or 18% Tris/glycine gels (Novex and Invitrogen), and transferred to polyvinylidene difluoride membrane. For FL-APP, C99, and C50 detection, blots were probed with anti-APP (676–685) and anti-Aβ (1:5000). In addition, FL-APP was also detected by immunoblot analysis using either antibody 22C11 (1:10,000) or 6E10 (1:10,000; which predominantly detects FL-APP in cell lysates). For detection of APPα and APPβsw, blots were probed with antibody 6E10 (1:10,000) and anti-APPβsw antisemur (1:5000), respectively. For β-actin detection, blots were probed with antibody A19 (1:250), and antibody against tubulin III (1:250). Following application of peroxidase-conjugated secondary antibodies, immunoblot signals were detected by enhanced chemiluminescence using ECL Plus and quantified using a Kodak Image Analyzer (440 CF).

RESULTS

Experimental Strategy for Analyzing Cholesterol-dependent Versus Isoprenoid-dependent Effects on APP Processing—Total cellular cholesterol levels are maintained by a combination of cholesterol biosynthesis in the endoplasmic reticulum (ER) (Fig. 1A) and receptor-mediated endocytosis of cholesterol-containing lipoprotein particles (reviewed in Refs. 54 and 55). In the event that one of these two cholesterol pathways is blocked, the other pathway is capable of maintaining normal cholesterol levels within the cell. For example, if cholesterol biosynthesis is inhibited by treatment with statin drugs, then cells may obtain cholesterol via low density lipoprotein receptor-mediated uptake of lipoprotein particles from the medium. Conversely, if the medium is deficient in lipoprotein particles, then cells may generate cholesterol endogenously through the biosynthetic pathway involving HMG-CoA reductase.

In the cholesterol biosynthetic pathway, statin-induced inhibition of HMG-CoA reductase blocks the synthesis of mevalonate and all downstream products, including both cholesterol and the nonsterol isoprenoids (Fig. 1A). As noted previously, the major cholesterol-independent effects of the statins are likely due to inhibition of isoprenoid pathways (30, 31). Studies have shown that statin-induced blockade of isoprenoid biosynthesis may be abrogated by adding low concentrations (~0.25 mM) of mevalonate to the culture medium during statin treatment (34, 37, 38, 52, 54–56). Importantly, under these conditions of statin plus low mevalonate, cholesterol production remains insignificant, whereas isoprenoid function is rescued.

We have exploited these properties of the cholesterol and isoprenoid pathways to develop an experimental strategy for manipulating cellular cholesterol and isoprenoid levels independently of one another (Fig. 1, B–F). With this strategy, we have been able to analyze the cholesterol-dependent versus isoprenoid-dependent effects of statin treatment on APP processing in cells. As a model cell culture system, we used HER293 cells stably transfected with human APP695 containing the Swedish mutation (APPsw-293 cells). APPsw-293 cells are technically facile and have been used extensively to analyze APP processing. We investigated statin effects under four conditions: Condition 1, normal cholesterol and low isoprenoid levels (Fig. 1C); Condition 2, low cholesterol and low isoprenoid levels (Fig. 1D); Condition 3, normal cholesterol and normal isoprenoid levels (Fig. 1E); and Condition 4, low cholesterol and normal isoprenoid levels (Fig. 1F). In Condition 1, cells are treated with statins in medium containing FBS, which is rich in cholesterol-containing lipoprotein particles (Fig. 1C), so both cholesterol and isoprenoid biosynthesis are blocked, but normal cellular cholesterol levels are maintained by low density lipoprotein receptor-mediated endocytosis. On the other hand, cells cultured with statins in LDLFBS (Condition 2) (Fig. 1D) cannot obtain cholesterol by endocytosis, and they are not capable of synthesizing cholesterol or isoprenoids due to HMG-CoA reductase inhibition. In this case, cells experience depressed levels of both cholesterol and isoprenoids. In Condition 3, supplementation of statin-treated cells with 0.25 mM meva-
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lonate in FBS-containing medium circumvents the statin-induced blockade of isoprenoid synthesis, and cells maintain normal cholesterol levels by endocytosis of FBS-derived lipoprotein particles (Fig. 1E). In Condition 4, statin-treated cells in LDFBS plus 0.25 mM mevalonate have normal isoprenoid levels due to mevalonate supplementation, but do not have a source of cholesterol from the medium and therefore experience depressed cholesterol levels (Fig. 1F). As a complementary method to examine the effects of cholesterol reduction on APP processing, total cellular cholesterol levels were lowered by treating cells with MβCD, which has been shown to selectively and rapidly extract cholesterol from the plasma membrane in preference to other lipids (34, 57).

To verify that we could independently manipulate cellular cholesterol and isoprenoid levels with our experimental strategy, APPsw-293 cells were treated according to the conditions outlined above, and total cellular cholesterol levels were measured using a cholesterol assay. As expected from previous studies (34, 37, 52), cell viability following treatment under all conditions was >95% as determined by trypan blue staining (data not shown). Exposure of APPsw-293 cells to either 10 mM MβCD for 30 min or 10 μM LV for 24 h in LDFBS-containing
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FIG. 3. Statin treatments induce the accumulation of cell-associated APP and β-secretase-cleaved APP fragments in an isoprenoid-dependent manner. APPsw-293 cells were treated with vehicle (control (C)) or the indicated concentrations of LV (A–E) or SV (F) for 48 h in FBS (A–C, and F, upper panel; and D, E, and F, middle and lower panels). Some cultures were supplemented with 0.25 mM mevalonate (MEV) (B and E) or 10 μM GGPP (C). Following treatment, cell lysates were prepared and analyzed by immunoblotting with anti-APP(676–695) antiserum plus anti-actin antibody (A–F, upper panels), antibody 22C11 (FL-APP) (A–F, middle panels; and I, upper panel), anti-APPβsw/APPsw antiserum (A–F, lower panels), or antibody 6E10 (I, lower panel). Note that LV and SV treatments caused a dose-dependent increase in the levels of cell-associated FL-APP and β-secretase-cleaved APP fragments and that supplementation with mevalonate or GGPP inhibited these increases. Similar effects were observed for 24-h treatments (not shown). For the identification of APPβsw and C83 and C99 CTFs, lanes in F were loaded with cell lysates from APPsw adenovirus-infected mouse neurons with wild-type (+) or BACE1−/− (−) genotypes. Comparison of the + and − lanes confirmed the identity of APPβsw (F, lower panel) and C99 (upper panel, + lane; −12-kDa band). Molecular mass markers (in kilodaltons) are shown on the right, as are the positions of mature (M) and immature (IM) APP. Under the lower panels in A–F are the conditions (Con.) described under “Results.” Normo, normal; Chol, cholesterol; Iso, isoprenoids. The immunoblots for the LV experiment were scanned on a...
for APP only. Because antibody 6E10 showed similar dose-dependent increases in immunoreactivity compared with antibody 22C11 and anti-APP-(676–695) antiserum, we concluded that signals from all three anti-APP antibodies predominantly represented levels of FL-APP rather than APP-like protein.

Both LV and SV appeared to have similar dose-response effects on FL-APP accumulation in cells maintained in either FBS or LDFBS (Fig. 3, compare A and D with F). Interestingly, the dose-response nature of statin treatment on APP levels contrasted with the absence of a dose-response relationship of statin treatment with total cholesterol levels (Fig. 2, C and D). Furthermore, increased FL-APP levels were observed for statin-treated cells maintained in either FBS-containing medium (Fig. 3, A, F, and I) or LDFBS-containing medium (Fig. 3D), indicating that the statin-induced accumulation of APP occurred regardless of whether total cellular cholesterol levels were normal (FBS; Condition 1) or low (LDFBS; Condition 2). As we will demonstrate below, these effects are not related to cellular cholesterol levels, but instead are due to low levels of isoprenoids in the cell.

Using the antibody 22C11 immunosignal for quantitation, at the highest LV dose (10 μM), the value of the FL-APP level reached ~400% of that found in vehicle-treated control cells (Fig. 3G). The anti-APP antibodies detected bands on immunoblots of ~110 and ~130 kDa that have been identified previously as immature APP and mature APP, respectively (59, 60). Notably, statin treatment appeared to elevate the level of immature APP to a greater extent than that of mature APP (FL-APP) (Fig. 3, A, D, and I), suggesting that the bulk of APP accumulation occurred within early biosynthetic compartments.

Our results suggested that statin treatment caused the post-translational accumulation of APP within the cell, although it was also possible that the increased APP levels may have been due to statin-stimulated up-regulation of APP transgene expression or APP mRNA stabilization. To test this hypothesis, we performed quantitative reverse transcription-PCR using APP-specific primers to amplify RNA isolated from LV-treated and control APPsw-293 cells. We found that APP mRNA levels in statin-treated cells were similar to those found in vehicle control cells (data not shown), demonstrating that the increase in mRNA was not due to an effect of statins on APP transgene expression or mRNA stability.

Immunoblot analysis with anti-APP-(676–695) antiserum also identified the α- and β-secretase-cleaved CTFs of APP, C83, and C99, respectively. We observed that the levels of C99 (~12 kDa) increased with LV and SV treatment in a dose-dependent manner (Fig. 3, A, D, and F, upper panels), thus paralleling the rise in FL-APP levels. This C99 increase occurred regardless of whether cells were treated in FBS-containing medium (Fig. 3, A and F) or LDFBS-containing medium (Fig. 3D). In contrast, the level of C83 (~10 kDa) in FBS-grown cells was not substantially raised following treatment (Fig. 3, A and F), whereas C83 levels were significantly elevated over control levels in statin-treated LDFBS-maintained cells (Fig. 3D). Interestingly, this C83 increase had approximately the same value for all LV doses (Fig. 3D) and was inversely correlated with the dose-independent decrease in total cholesterol levels following LV treatment in LDFBS (Fig. 2, C and D). In addition to the rise in C99 and C83 levels, statin treatment also increased the levels of at least six unidentified APP C-terminal immunoreactive bands ranging in size from ~15 to ~180 kDa on blots incubated with anti-APP-(676–695) antiserum (Fig. 3, A, D, and F). Further investigation will be required to fully characterize these additional APP-derived CTFs.

The elevated level of C99 implied that the other β-secretase cleavage product, APPsβ, might also be increased following statin treatment. To investigate this possibility, we immunoblotted the lysates from statin-treated APPsw-293 cells and incubated the blots with antiserum directed against the free C terminus of APPsβsw generated after β-secretase cleavage. This neoepitope antibody has high affinity for the cleaved APPsβsw ectodomain, but only very weakly cross-reacts with FL-APPsw (Fig. 3F, lower panel, compare + and −lanes). As predicted from the rise in C99 levels, immunoblot analysis of cell lysates using anti-APPsβsw antiserum revealed that levels of cell-associated APPsβ increased in a dose-dependent manner following exposure to either LV or SV (Fig. 3, A, D, and F, lower panels). Moreover, the statin-induced APPsβsw increase was dramatic, reaching ~2500% of control cell levels for 10 μM LV (Fig. 3H), and occurred regardless of whether cells were treated in FBS- or LDFBS-containing medium. The apparent accumulation of APPsβ within the cell is atypical because APPsβ is normally efficiently secreted into the extracellular medium, and very little APPsβ remains cell-associated.

To directly establish whether the large increase in cell-associated APPsβ detected by immunoblotting was the result of APPsβsw accumulation within intracellular compartments, we performed indirect immunofluorescence microscopy on LV-treated APPsw-293 cells following incubation with anti-APPsβsw antiserum (Fig. 4B). As expected, a robust anti-APPsβsw signal was observed within subcellular compartments in statin-treated cells (Fig. 4B), demonstrating that the increase in the levels of cell-associated APPsβ was indeed the result of intracellular accumulation.

The high levels of amyloidogenic C99 and intracellular APPsβ indicated that β-secretase cleavage of APP increased in cells following statin treatment, and therefore, it appeared likely that Aβ levels could also be elevated in response to statins. To investigate this possibility, we treated APPsw-293 cells with LV in FBS or LDFBS as described above and analyzed cell lysates for levels of intracellular Aβ40 and Aβ42 by specific sandwich ELISAs. As observed for the levels of FL-APP and β-secretase-cleaved APP fragments, statin treatment in either FBS- or LDFBS-containing medium caused a dose-dependent increase in Aβ40 levels within the cell (Fig. 5, A–C). Cell-associated Aβ42 was below the level of detection in the Aβ42-specific ELISA for the doses and times of LV treatment tested (data not shown). We also noted that untreated cells maintained in LDFBS exhibited cell-associated Aβ40 levels that were slightly lower than those observed in cells maintained in FBS. In addition, the LV-induced increases in cell-associated Aβ40 levels appeared muted in cells maintained in LDFBS compared with those in FBS (Fig. 5, A and B). However, following normalization of Aβ40 levels to cellular protein content, the magnitudes of the cell-associated Aβ40 increases in cells grown in either FBS or LDFBS were approximately the same, reaching
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Fig. 4. Statin causes the accumulation of APPsβ within intracellular compartments. APPsw-293 cells were exposed to vehicle (Control), 10 μM LV for 48 h in FBS, or 10 mM MβCD for 30 min. Following treatment, cells were fixed and sequentially incubated with anti-APPsw antisera and fluorescein 5-isothiocyanate-conjugated goat anti-rabbit antibodies to visualize intracellular APPsw (green). Note the profound accumulation of APPsβ within intracellular compartments in LV-treated APPsw-293 cells. MβCD treatment showed the opposite effect, with reduced intracellular APPsβ immunoreactivities compared with the control. Scale bars = 10 μm.

~400% of control values for the 10 μM doses (Fig. 5C).

Taken together, our results thus far demonstrated that statin treatments induced the accumulation of APP, C99, APPsβ, and Aβ40 within cells in a dose-dependent manner. The cell-associated increases in these species occurred regardless of whether cells were treated in medium containing FBS (Condition 1, normal cholesterol and low isoprenoid levels) or LDFBS (Condition 2, low cholesterol and low isoprenoid levels). In addition, C83 levels increased markedly following statin treatment of LDFBS-maintained cells and were inversely correlated with cellular cholesterol levels. However, most importantly, our data suggest that low cellular isoprenoid levels may be responsible for increased β-secretase cleavage and intracellular accumulation of C99, APPsβ, and Aβ and that cellular cholesterol levels may not play a significant role in mediating these effects.

Mevalonate Supplementation Rescues Statin-induced Accumulation of Cell-associated Amyloidogenic Fragments by Providing for GGPP Biosynthesis—Previous studies have demonstrated that supplementation of statin-treated cells with 0.25 mM mevalonate rescues isoprenoid production without significantly increasing cholesterol biosynthesis (34, 37, 38, 52, 54–56). Therefore, to investigate the role of the isoprenoids in the statin-induced buildup of APP, C99, APPsβ, and Aβ40 within cells, we treated APPsw-293 cells with different concentrations of LV in the presence of 0.25 mM mevalonate in FBS (Condition 3, normal cholesterol and normal isoprenoid levels) or LDFBS (Condition 4, low cholesterol and normal isoprenoid levels). We then harvested cell lysates for immunoblot analysis and Aβ ELISAs as described above and compared the results with those obtained for LV-treated cells in FBS (Condition 1, normal cholesterol and low isoprenoid levels) or LDFBS (Condition 2, low cholesterol and low isoprenoid levels). Application of 0.25 mM mevalonate alone to either FBS- or LDFBS-maintained cells had no apparent effect on the levels of cell-associated and secreted APP or APP-derived fragments (data not shown).

Supplementation of LV-treated cells with 0.25 mM mevalonate in FBS-containing medium completely prevented the statin-induced increases in the levels of cell-associated FL-APP, APPsβ, and C99 and the other APP-derived fragments that were observed following treatment with LV alone (Fig. 3, compare A with B; G and H). In addition, cell-associated Aβ40 levels in statin-treated cells were reduced to control values by mevalonate supplementation (Fig. 5, A–C). This rescue of statin-induced increases by 0.25 mM mevalonate under normal cholesterol conditions (FBS) provides further evidence that isoprenoids affect intracellular levels of β-secretase-cleaved APP fragments and Aβ in a cholesterol-dependent manner.

Next, we wanted to determine the effects of mevalonate supplementation on statin-treated cells in a low cholesterol environment (LDFBS). Similar to the results observed with FBS plus mevalonate, 0.25 mM mevalonate in LDFBS-containing medium greatly inhibited statin-induced increases in the levels of C99 and cell-associated APPsβ (Fig. 3, compare D and E; F, H). Moreover, intracellular Aβ40 levels were equivalent to vehicle control levels following mevalonate supplementation in LDFBS (Fig. 5, A–C). These results again indicate that low cellular isoprenoid levels promote β-secretase cleavage and the intracellular accumulation of Aβ and β-secretase-cleaved fragments, whereas cellular cholesterol levels do not appear to play a predominant role.

In contrast to mevalonate supplementation in FBS, LDFBS plus 0.25 mM mevalonate did not completely block the statin-induced accumulation of FL-APP as determined by immunoblot analysis with anti-FL-APP antibodies (Fig. 3, E and G). Mevalonate supplementation in LDFBS did, however, blunt the FL-APP increase at the lower LV doses (Fig. 3, compare D and E; F, G). The failure of mevalonate supplementation to completely rescue the statin-induced buildup of FL-APP in LDFBS (low cholesterol and normal isoprenoid levels) (Fig. 3E) suggests that the increase in cell-associated APP may be partly due to low cellular cholesterol levels because FBS plus 0.25 mM mevalonate (normal cholesterol and normal isoprenoid levels) (Fig. 3B) prevented the FL-APP increase. However, isoprenoids must also influence FL-APP levels because statin-induced APP accumulation occurred in FBS (normal cholesterol and low isoprenoid levels) (Fig. 3A), although it was blunted at low LV concentrations (Fig. 3G). Thus, FL-APP levels in the cell appear to be affected by both cholesterol-dependent and isoprenoid-dependent mechanisms.

We also observed that C83 levels did not substantially change in cells treated with LV in LDFBS plus 0.25 mM mevalonate (Fig. 3E) compared with cells exposed to LV alone in LDFBS (Fig. 3D). In contrast, as already noted, C99 levels were dramatically reduced by addition of mevalonate. Because cells grown in statin plus LDFBS-containing medium had low cellular cholesterol levels regardless of mevalonate supplementation, but varied with respect to their isoprenoid levels, a low cholesterol environment appears to favor α-secretase processing of APP, whereas isoprenoids may have less influence on non-amyloidogenic cleavage.

Mevalonate is an upstream precursor of cholesterol and the nonsterol isoprenoids FPP and GGPP (Fig. 1A). Importantly, many cholesterol-independent statin effects may be caused by reduced levels of GGPP (reviewed in Ref. 31). To specifically determine the role of GGPP in the isoprenoid-dependent accumulation of FL-APP, APPsβ, C83, and C99 in the absence of changes in total cholesterol, we supplemented LV-treated cells with 10 μM GGPP in FBS (normal cholesterol and normal GGPP levels) (Fig. 3C) and compared the effects on APP metabolism with those obtained following LV treatment alone in
FBS (Fig. 3A). Similar to the results observed with mevalonate supplementation (Fig. 3B), 10 μM GGPP completely prevented the statin-induced increases in the levels of cell-associated FL-APP, APPβ40, and C99 and the other APP-derived fragments (Fig. 3, compare C with A). These results demonstrate that low levels of the isoprenoid GGPP are the major cause of the cell-associated accumulation of amyloidogenic fragments during statin treatment.

In summary, our results with mevalonate supplementation further support the view that low cellular isoprenoid levels enhance β-secretase cleavage and the accumulation of intracellular Aβ and β-secretase-cleaved APP fragments. Furthermore, using GGPP supplementation, we have conclusively demonstrated that the statin-induced accumulation of amyloidogenic fragments is specifically due to the suppression of GGPP biosynthesis. Conversely, low cellular cholesterol levels increase processing in the non-amyloidogenic α-secretase pathway. Finally, both low cholesterol and low isoprenoid levels appear to act additively to increase FL-APP levels in cells.

Low Cellular Cholesterol Levels Enhance and Suppress the α- and β-Secretase Pathways, Respectively—Our work thus far focused on the effects of cholesterol and isoprenoids on cell-associated APP fragments and Aβ, but we were also interested in determining how statin treatments in our system affect secreted APP products, i.e. APPαo, APPβ, and Aβ. Therefore, we treated APPsw-293 cells with LV in FBS or LDFBS with or without 0.25 mM mevalonate and analyzed the conditioned media for APPαo and APPαβ by immunoblotting and for Aβ40 and Aβ42 by specific ELISAs. To detect APPαo, we incubated immunoblots with antibody 6E10, which recognizes Aβ-(1–17), which is present at the C terminus of APPαo. In FBS-containing medium, neither APPαo nor APPαβ levels changed significantly following exposure to statin compared with the vehicle control levels (Fig. 6, A and E). On the other hand, the medium from LV-treated cells grown in LDFBS showed a
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consistent ~2-fold increase in APPsα levels, but APPsβ levels remained at control values (Fig. 6, C and F). Like the increase in C83 levels observed for LV-treated cells in LDFBS (Fig. 3, D and E), the levels of APPsα in conditioned medium reached maximum values at the lowest LV dose tested (1 μM) (Fig. 6F). Mevalonate supplementation did not significantly alter the levels of either APPsα or APPsβ in conditioned medium compared with mevalonate-minus medium (Fig. 6, compare A with B and C with D). These results clearly show that the increase in APPsα levels in the medium is associated with low cellular cholesterol levels (because it occurred exclusively in LDFBS) and is unrelated to isoprenoid levels (because mevalonate supplementation had no effect). The lack of any change in APPsβ secretion into the medium, regardless of isoprenoid status, was unexpected given the strong statin-induced increase in intracellular APPsβ with low isoprenoid levels (Fig. 3, A, D, and F).

Because a low cholesterol environment appears to enhance the non-amyloidogenic α-secretase pathway, Aβ secretion should decrease under conditions that lower total cellular cholesterol levels in our system. To test this hypothesis, we measured Aβ40 and Aβ42 in the conditioned medium from the same LV-treated APPsw-293 cells that were analyzed for secreted APPsα and APPsβ. As reported previously, the total amount of secreted Aβ40 was ~10-fold higher than the total amount of secreted Aβ42 in all samples (−400 and −40 ng, respectively) (Fig. 5, D and G). In addition, the levels of secreted Aβ40 and Aβ42 were significantly reduced in untreated cells maintained in LDFBS compared with cells maintained in FBS (Fig. 5, D, E, G, and H). The reduction of secreted Aβ levels in LDFBS was not due to decreased FL-APP levels because vehicle control APPsw-293 cells had similar levels of FL-APP in both LDFBS and FBS as determined by antibody 22C11 immunoblot analysis (Fig. 3J). Most importantly, we observed that the levels of both secreted Aβ40 and Aβ42 were reduced to ~50% of control values following treatment with LV in LDFBS either with or without mevalonate supplementation (Fig. 5, D–I). In contrast, secreted Aβ40 and Aβ42 levels from LV-treated cells in FBS were not significantly different from control levels. Thus, the decrease in the levels of Aβ40 and Aβ42 secreted into the medium was strongly associated with low cellular cholesterol levels (Conditions 2 and 4) and did not correlate with isoprenoid status in cells. In this regard, secreted Aβ40 and Aβ42 on the one hand and secreted APPsα on the other exhibited cholesterol-dependent and isoprenoid-independent behaviors, although the respective levels of secreted Aβ and APPsα changed in opposite directions following treatment.

To confirm by another method that low cellular cholesterol levels enhance the α-secretase pathway and thereby indirectly suppress β-secretase processing, we treated APPsw-293 cells with 10 mM MβCD and performed immunoblot analysis on cell lysates for C83 and APPsβ. Unlike statin treatment, which inhibits de novo cholesterol synthesis in the ER, MβCD preferentially removes cholesterol from the plasma membrane (34, 37, 57). As observed for statin-treated cells maintained in LDFBS (Fig. 3, D and E), exposure to MβCD resulted in an increase in C83 levels (Fig. 7A). However, in contrast to statin treatment, MβCD reduced the level of cell-associated APPsβ to
levels with MβCD harvested and analyzed by immunoblotting with anti-APP-(676–695) antiserum (FL-APP and C83), anti-APPsβsw antiserum (APPsβ), antibody 6E10 (APPsα), and anti-actin antibody (loading control). The immunoblots were scanned on a PhosphorImager, quantified, and normalized against actin signal (cell lysates) or total cellular protein concentration (media) and are expressed as a percentage of the control ± S.E. (B and D). Note that lowering the total cellular cholesterol levels with MβCD (MβtCD) caused an increase in the levels of α-secretase cleavage products C83 (A) and APPsα (C) and a decrease in the levels of intracellular APPsβ (A and B). A slight increase in FL-APP levels was also observed following MβCD treatment (A and B). Similar effects were observed for cells maintained and treated in LDFBS (not shown).

~40% of control values (Fig. 7, A and B) and visibly decreased intracellular APPsβ accumulation as determined by anti-APPsβsw immunofluorescence microscopy (Fig. 4C). The levels of FL-APP appeared slightly increased by MβCD treatment in our system (Fig. 7, A and B), in agreement with our previous conclusion that low cellular cholesterol contributes to elevated FL-APP levels (Fig. 3E).

Next, we measured APPsα and APPsβ levels in the conditioned medium from MβCD-treated cells. Similar to the effects of statin treatment in LDFBS-containing medium (Fig. 6, C and D), we observed an elevation of APPsα levels in the medium compared with control levels and detected no change in APPsβ levels (Fig. 7, C and D). The increase in secreted APPsα mirrored the rise in C83 levels observed following exposure to MβCD (Fig. 7A). It is notable that the magnitude of the APPsα increase was large (~600% of control values) and exceeded that observed for LV-treated cells in LDFBS (Fig. 6, C, D, and F). The more robust APPsα secretion may be related to the greater ability of MβCD to lower total cellular cholesterol levels compared with statin treatment in LDFBS (Fig. 2). In any case, taken together, our results with both LV treatment in LDFBS and MβCD treatment demonstrate that low cellular cholesterol levels enhance α-secretase processing of APP, as indicated by higher levels of C83 and secreted APPsα, and suppress the β-secretase pathway, as shown by lower levels of intracellular APPsβ (MβCD) (Fig. 7A) and secreted Abβ (LV in LDFBS) (Fig. 5, D–I).

**Statins Induce the Accumulation of Cell-associated β-Secretase-cleaved APP Fragments in Neural and Astrocyte Cells**—Although HEK293 cells have been widely used to study APP processing, they are not of central nervous system origin and therefore may not exhibit the same statin-induced effects as those of physiologically relevant cell types involved in AD. To address this concern, we statin-treated several neural and astrocyte cell types, such as human SH-SYSY neuroblastoma cells (A) and mouse Tg2576 primary cortical neurons (B and C) were treated with vehicle (control (C)) or the indicated concentrations of LV for 48 h. Prior to LV exposure, SH-SYSY cells were infected with APPsw adenovirus for 48 h. Following LV treatment, cell lysates were prepared and analyzed by immunoblotting with anti-APP-(676–695) antiserum (FL-APP, C99, and C83), anti-APPsβsw antiserum, anti-BACE1 antibody, and anti-actin antibody. Cell-associated FL-APP levels and cleaved APP fragments were increased in SH-SYSY cells by LV (A). For primary neurons, FL-APP immunoblot signals were scanned on a PhosphorImager, quantified, and normalized against actin and are presented as a percentage of the control ± S.E. (C). Note that LV treatment increased the levels of both FL-APP and BACE1 in primary neurons (B).

**FIG. 7. Reduced cellular cholesterol favors α-secretase over β-secretase processing of APP.** APPsw-293 cells were treated with vehicle (control (C)) or 10 mM MβCD for 30 min in FBS-containing medium, and then cell lysates (A and B) and media (C and D) were harvested and analyzed by immunoblotting with anti-APP-(676–695) antiserum (FL-APP and C83), anti-APPsβsw antiserum (APPsβ), antibody 6E10 (APPsα), and anti-actin antibody (loading control). The immunoblots were scanned on a PhosphorImager, quantified, and normalized against actin signal (cell lysates) or total cellular protein concentration (media) and are expressed as a percentage of the control ± S.E. (B and D). Note that lowering the total cellular cholesterol levels with MβCD (MβtCD) caused an increase in the levels of α-secretase cleavage products C83 (A) and APPsα (C) and a decrease in the levels of intracellular APPsβ (A and B). A slight increase in FL-APP levels was also observed following MβCD treatment (A and B). Similar effects were observed for cells maintained and treated in LDFBS (not shown).

**FIG. 8. Statin induces the accumulation of cell-associated APP and APP fragments in neural cells.** Human SH-SYSY neuroblastoma cells (A) and mouse Tg2576 primary cortical neurons (B and C) were treated with vehicle (control (C)) or the indicated concentrations of LV for 48 h. Prior to LV exposure, SH-SYSY cells were infected with APPsw adenovirus for 48 h. Following LV treatment, cell lysates were prepared and analyzed by immunoblotting with anti-APP-(676–695) antiserum (FL-APP, C99, and C83), anti-APPsβsw antiserum, anti-BACE1 antibody, and anti-actin antibody. Cell-associated FL-APP levels and cleaved APP fragments were increased in SH-SYSY cells by LV (A). For primary neurons, FL-APP immunoblot signals were scanned on a PhosphorImager, quantified, and normalized against actin and are presented as a percentage of the control ± S.E. (C). Note that LV treatment increased the levels of both FL-APP and BACE1 in primary neurons (B).

Next, we cultured primary cortical neurons isolated from transgenic mice expressing APPsw95sw (Tg2576) (61) and treated them with 7.5 μM LV for 48 h. The purity of our neuronal cell cultures was ~95% as determined by β-tubulin III immunofluorescence microscopy (data not shown). Immunoblot analysis of lysates from LV-treated Tg2576 neurons using anti-APP-(676–695) antiserum revealed that FL-APP levels were increased to ~500% of control Tg2576 neuron values (Fig. 8, B and C), which is similar in magnitude to the APP increases observed in statin-treated APPsw-293 cells (Fig. 3G).

It is interesting to note that, in most cell types we analyzed, statin treatment appeared to elevate the level of C99 to a
FBS-containing medium for either 48 h (logical activation. and APP fragments in astrocytes and appear to cause morpho-

tures of astrocytes isolated from Tg2576 mice with different
term of this study was the development of an effective in vitro system that would enable the discrete analysis
of specific statin effects on APP metabolism, and to this end, a robust non-neuronal HEK293 cell type was employed.
In addition, we examined statin effects on APP metabolism in AD-relevant neural cell types. Importantly, statins elevated intracellular levels of APP and β-secretase-cleaved fragments in all cell types analyzed. These effects were not due to statin-induced increases in APP transgene expression or mRNA stability. Moreover, the accumulation of cell-associated FL-APP and β-secretase-cleaved fragments occurred even at low, physiologically relevant (nanomolar) statin concentrations and thus was not an artifact of high drug doses.

Our results are consistent with a model in which low cellular isoprenoid levels inhibit the trafficking of APP through the secretory pathway. Assuming that the rates of APP protein synthesis and degradation remain unchanged, reduced transport of APP through the secretory pathway would lead to elevated levels of APP in biosynthetic compartments (i.e. ER, Golgi, and trans-Golgi network (TGN)) (Fig. 10). This would account for our observation that immature APP levels were increased upon statin inhibition of isoprenoid synthesis (Fig. 3, A, D, F, and I). Because the TGN is a major site of BACE1 intracellular localization (18), accumulation of APP in the TGN would raise rates of enzyme-substrate interaction and subsequent β-secretase cleavage of APP, thus increasing APPβ and C99 levels. This effect could be exacerbated by concomitant accumulation of BACE1, as suggested by our data in primary neurons (Fig. 8B). Finally, γ-secretase is also localized within the TGN (62), leading to increased conversion of C99 into Aβ and accumulation of intracellular Aβ.

In contrast, low cellular cholesterol levels do not appear to significantly inhibit outward transport of APP, but may instead reduce the rate of endocytosis of cell-surface APP, as suggested previously (38, 57). Thus, cell-surface APP levels would become elevated, and the rate of APP processing by α-secretase, which...
resides on the plasma membrane, would increase. This hypothesis is supported by the increased levels of C83 and APPsβ that we (Fig. 7, A, C, and D), and others (37, 38) have observed under low cellular cholesterol conditions. Moreover, a cholesterol-dependent decrease in the rate of APP endocytosis would reduce the levels of APP in endosomal compartments, also sites of BACE1 and γ-secretase localization, and would thus lead to lower levels of Aβ production and secretion, as this study and others have shown (34, 37, 38).

**Independent Intracellular and Secreted Pools of Aβ**—We have provided evidence for two cellular pools of Aβ (one intracellular and the other secreted) that behave independently of one another. For example, statin-treated cells in PBS (Condition 1, normal cholesterol and low isoprenoid levels) or LDFBS (Condition 2, low cholesterol and low isoprenoid levels) both accumulated intracellular Aβ to levels that were ~4-fold above control values, yet Condition 1 cells secreted control levels of Aβ, whereas Condition 2 cells secreted only about half that amount (Fig. 5). Conversely, cells grown in LDFBS plus mevalonate (Condition 4, low cholesterol and normal isoprenoid levels) had control levels of intracellular Aβ (unlike Condition 2 cells) and secreted low levels of Aβ (similar to Condition 2 cells). Thus, the intracellular Aβ pool is affected by isoprenoids, whereas the secreted Aβ pool is influenced by cholesterol.

We noted that the amount of total intracellular Aβ40 was small compared with that of total extracellular Aβ40 (e.g., ~0.45% for 10 μM statin-treated LDFBS cells) (Fig. 5, A and D). However, comparisons of total Aβ levels in the medium versus cell lysates are not true indications of the relative rates of Aβ production in the two compartments because the respective rates of Aβ degradation in the medium versus the cell are unknown and may differ widely. In fact, it is possible that the production rates of secreted and intracellular Aβ may be similar, but Aβ degradation may be more rapid within the cell than in the medium, leading to the accumulation of greater absolute amounts of secreted Aβ compared with intracellular Aβ. Therefore, comparisons of total Aβ amounts have the potential to be misleading and may lead to the false impression that cell-associated Aβ production is not significant. Indeed, our estimate of Aβ40 concentrations within the cell (total cell-associated Aβ40 divided by the cell pellet volume) (Fig. 5B) is significant (e.g., ~45% of the media Aβ40 concentration for 10 μM statin-treated LDFBS cells) (Fig. 5E). Moreover, we predict much higher Aβ concentrations within the intracellular compartments in which Aβ accumulation occurs. The major findings of this study are that intracellular Aβ levels increase dramatically upon statin-induced inhibition of isoprenoid synthesis and that distinct pools of intracellular and secreted Aβ exist that are largely isoprenoid-dependent and cholesterol-dependent, respectively. Although the consequences of high in-
tracellular $\beta$ concentrations are not fully understood, mounting evidence suggests that accumulation of intraneuronal $\beta$ may play an early role in AD pathogenesis (5–15). Thus, we suggest that the isoprenoid-dependent accumulation of cell-associated $\beta$ represents a significant finding that may have important implications for mechanisms of AD.

It should be noted that we cannot currently completely exclude the possibility that low isoprenoid levels cause a shift from $\beta$ secretion to intracellular accumulation. However, because low isoprenoids do not lead to decreases in the levels of secreted $\beta$ or APPs $\beta$ (which is assumed to parallel $\beta$), then a shift from secretion to accumulation would have to be relatively small and not result in any detectable reductions in secreted $\beta$ or APPs $\beta$. We suggest that this scenario is unlikely because the dramatic isoprenoid-dependent increase in the levels of cell-associated $\beta$ and APPs $\beta$ that we observed would be expected to be associated with decreases in secreted $\beta$ and APPs $\beta$ levels if a significant shift from secretion to accumulation had occurred. Instead, our results support the hypothesis that low isoprenoid levels promote the accumulation of an intracellular $\beta$ pool without significantly affecting the secreted $\beta$ pool and are consistent with previous studies demonstrating an intracellular $\beta$ pool (63). To our knowledge, this study is the first to show that the distinct intracellular and secreted $\beta$ pools are differentially affected by cellular isoprenoid and cholesterol levels, respectively.

**Isoprenoid-dependent Mechanisms Underlying the Effects of Statins on APP Metabolism**—Our model in which low isoprenoid levels slow down APP transport through the secretory pathway is supported by evidence that inhibition of isoprenylation of key regulatory proteins involved in protein trafficking (e.g. G-proteins in the Rho and Rab families) is associated with cytoskeletal alterations and a decrease in the efficiency of vesicular transport (64, 65). In this study, statin-induced increases in intracellular APP and its metabolites were prevented by addition of mevalonate in a manner that did not significantly affect total cellular cholesterol levels. As detailed above, mevalonate is required for the biosynthesis of FPP and GGPP as well as cholesterol. GGPP predominantly functions to isoprenylate (geranylgeranylate) a large number of target proteins, whereas the number of farnesylated proteins is less (66–68). Furthermore, FPP is generally accepted to be the common branch point in the cholesterol pathway, being a metabolic precursor of both sterol (e.g. cholesterol) and nonsterol (e.g. GGPP) products. Here, the dependence of APP and amyloidogenic fragment accumulation on inhibition of nonsterol isoprenoid synthesis was established by the demonstration that these effects were fully rescued with GGPP supplementation, further suggesting the involvement of isoprenylated G-proteins. Indeed, previous studies have demonstrated that geranylgeranylated G-proteins such as Rab1B (69) and Rab6 (70) play an important role in the trafficking and processing of APP. Although further investigation is required to determine the identity of the geranylgeranylated target proteins responsible for the effects we have observed, to our knowledge, this study provides the first evidence that interference with isoprenoid synthesis causes the intracellular accumulation of amyloidogenic fragments and $\beta$.

As discussed above, the specifics of intracellular APP degradation (and associated fragments) have not been examined in this study. However, in addition to the accumulation of intracellular APP, APPs $\beta$, C99, and $\beta$ under low isoprenoid conditions, we noted significant buildup of several uncharacterized APP fragments of varying sizes (Fig. 3, A, D, and F). It is possible that these APP fragments are due to a degradation mechanism that rids the cell of excess proteins from intracellular compartments. Further investigation into the nature of these APP fragments is required.

Interestingly, statin treatment under conditions that led to the accumulation of APP and amyloidogenic fragments was dose-dependently coupled to significant alterations in astrocyte morphology, leading to an activated-appearing phenotype. These observations imply a statin-induced rearrangement of the astrocyte cytoskeleton. Recently, Bi et al. (32) reported that statins activate microglia through inhibition of isoprenoid biosynthesis. Because G-proteins in the Rho family are involved in regulating the organization of the actin cytoskeleton, it is possible that Rho may play a role in the cytoarchitectural changes that occur upon astrocyte activation. Whether the morphological changes observed in our study are linked to true physiological astrocyte activation remains to be determined.

**Cholesterol-dependent Mechanisms Underlying the Effects of Statins on APP Metabolism**—As we (this study) and others (34, 37, 57) have shown, total cholesterol levels are reduced by treatment with either statins or MβCD. It is well documented that statins and cyclodextrins lower cellular cholesterol levels via different mechanisms. Statins inhibit cholesterol biosynthesis, whereas MβCD specifically sequesters plasma membrane cholesterol to rapidly depress total cellular cholesterol levels (34, 37, 57). Kojro et al. (38) reported previously that more than one mechanism underlies the increase in non-amyloidogenic $\alpha$-secretase processing of APP following reduction of cellular cholesterol. They showed that impaired internalization of APP and increased membrane fluidity are responsible for increased $\alpha$-secretase cleavage after acute cholesterol depletion by MβCD, whereas LV stimulates APP$\alpha$ secretion by increasing the expression of ADAM10 (a disintegrin and metalloprotease). Although we did not analyze the specific mechanisms by which MβCD and statins increase the non-amyloidogenic processing of APP, it is possible that the observed statin-induced cholesterol-dependent increases in $\alpha$-secretase processing might be due in part to elevated ADAM10 levels or activity. In addition, the mechanistic differences in the cholesterol-lowering actions of the two drugs may have also contributed to the differential effects on APP processing that we observed.

In our experimental system, although we were able to modulate total cellular cholesterol levels efficiently and thus determine the effects of cholesterol on APP processing, we did not investigate in detail how cholesterol localization within the cell may influence APP cleavage and $\beta$ production. Cordy et al. (71) have demonstrated that localization of BACE1 to cholesterol-rich lipid rafts increases $\beta$ production and that raft disruption by cholesterol-lowering agents is closely associated with decreased amyloidogenic processing. Their work indicates that $\beta$ production occurs predominantly in cholesterol-rich rafts and that BACE1 is the rate-limiting enzyme involved in this process. Given these observations, the levels and subcellular distributions of cholesterol and BACE1 in relation to those of APP are expected to play a crucial role in $\beta$ production.

In this study, filipin staining of cholesterol, followed by immunofluorescence microscopy, did not reveal obvious redistributions of free intracellular cholesterol with any of our treatments (data not shown). However, these were low resolution studies, and we cannot exclude the possibility of subtle changes in cholesterol localization at the subcellular level following statin treatment. Indeed, we speculate that small differences in subcellular cholesterol levels or localization may account for the observation that altering the supply of exogenous cholesterol is associated with changes in total $\beta$ production. We noted that secreted $\beta$ levels were significantly lower in untreated cells maintained in LDFBS compared with those maintained in FBS (Fig. 5, D, E, G, and H). Although cells main-
tained in either FBS or LDFBS had equivalent total cholesterol levels in the absence of statins (Fig. 2), they differed in how low cholesterol levels in this compartment would be expected to reduce the production of secreted Aβ, as is consistent with our results (compare control secreted Aβ levels in FBS versus LDFBS) (Fig. 5, D, E, G, and H). The intracellular Aβ pool appears to be generated in biosynthetic compartments and thus may be less affected by low cholesterol in the endosomal pathway, which is consistent with our observation that intracellular Aβ levels were only slightly lower in control cells maintained in LDFBS compared with those maintained in FBS (Fig. 5, A and B). The extent to which cholesterol localization and lipid raft physiology are affected by cholesterol biosynthesis in the ER versus cholesterol uptake via receptor-mediated endocytosis remains to be determined.

Finally, other work has demonstrated that aberrant cholesterol trafficking (45) and high cholesterol easter levels (43) are associated with elevated γ-secretase activity and increased Aβ production. Clearly, further investigation into the effects of cholesterol trafficking, localization, and form on APP processing and Aβ generation is warranted.

Conclusion—Although the mechanisms by which statins exert beneficial effects in AD remain unclear, our in vitro experiments have established that cellular isoprenoid levels can affect the accumulation of intracellular Aβ and amyloidogenic fragments. Given recent evidence that intracellular Aβ may play a role in AD pathophysiology (5, 6, 8–15), it is critical to investigate the mechanisms of isoprenoid-dependent accumulation of intracellular Aβ and to identify the isoprenylated target proteins that are involved. Such studies may provide useful information for the discovery of novel therapeutic approaches for the treatment of AD.

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