

PHARMACOLOGICAL CONCENTRATIONS OF THE HMG-COA REDUCTASE INHIBITOR LOVASTATIN DECREASE THE FORMATION OF THE ALZHEIMER β -AMYLOID PEPTIDE *IN VITRO* AND IN PATIENTS

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1. ABSTRACT

Epidemiological studies demonstrate that hypercholesterolemia is a risk factor for Alzheimer's disease (AD). As the generation and accumulation of the β -amyloid peptide (β A) in the brain appears to be significant for the initiation and progression of AD, it is possible that cholesterol levels regulate β A formation and/or clearance. To test the effects of altering cholesterol on β A formation, we incubated cells with or without lovastatin acid, the active metabolite of the HMG-CoA reductase inhibitor lovastatin, and measured the fraction of β A formed from its precursor under each condition. We observed that treatment with lovastatin acid led to a profound decrease in the levels of β A formed. This effect was observed at concentrations of 0.05-5 μ M, ranges where this compound is effective at inhibiting HMG-CoA reductase. To examine the effects of

lovastatin on β A *in vivo*, human subjects who had elevated low-density lipoprotein cholesterol were treated during a double-blind, randomized study with 10-60-mg once-daily doses of a controlled-release formulation of lovastatin, or matching placebo. Serum β A concentrations were measured before and after up to 3 months of treatment. Mean and median changes from baseline in serum β A concentrations showed a significant ($p \leq 0.0348$), dose-dependent decrease. Differences between the 40- and 60-mg dose groups and placebo were statistically significant (Dunnett's $p < 0.05$). Our results suggest a mechanism by which hypercholesterolemia may increase risk for AD and indicate that lovastatin reduces β A formation and may thereby be effective in delaying the onset and/or slowing the progression of AD.

2. INTRODUCTION

AD is characterized by the presence of amyloid plaques that accumulate in the brain (reviewed in 1). Amyloid plaques have at their core a proteinaceous deposit comprised almost exclusively of the A β peptide. The A β peptide is a 40-43 amino acid long peptide that can aggregate *in vitro* and *in vivo* to form fibrils. A β is derived by proteolysis of the Alzheimer A β protein precursor (APP), which is a type I transmembrane glycoprotein that has a large extracellular domain and a short cytoplasmic domain (reviewed in 2). APP can be cleaved by 3 proteases, called α -, β -, and γ - secretases (see ref 2. and figure 2). α -Secretase cleaves APP within the A β domain releasing a large COOH-terminally truncated, secreted form of APP called APP_s. Because α -secretase cleaves within the A β domain, action of this protease precludes the formation of A β . β -Secretase cleaves APP at the amino terminus of the A β domain. When this protease activity is followed by γ -secretase, which cleaves at the carboxyl-terminal of the A β domain, A β is formed.

There is much evidence to suggest that the formation and accumulation of A β is critical for the initiation and progression of AD (reviewed in 3). Very rare forms of familial AD are caused by mutations in APP that lead to increased formation of A β . Similarly, more common forms of familial AD are caused by mutations in the presenilins. Mutations in the presenilins all lead to increased formation of A β . A major genetic risk factor for non-familial AD is the presence of the E4 allele of apolipoprotein E. Apolipoprotein E, which binds A β *in vitro* and can regulate its clearance (4-7), has been shown to be involved in the accumulation of A β in transgenic animals in an allele-dependent fashion with the E4 allele being most potent (8). Genetic risk factors for AD therefore increase A β formation and/or alter A β clearance. For this reason it has been assumed and that A β formation and/or the failure to clear A β is an initiating event in the disease. This is supported by studies in clinical samples. For example, in recent studies measuring either total amyloid load or total A β in postmortem brain samples, the levels of amyloid and A β correlated with cognitive decline (9-12). Furthermore, in these studies, amyloid load and total A β were elevated at the earliest signs of cognitive decline, before there would necessarily be a diagnosis of AD.

Epidemiological studies indicate that cholesterol levels represent a significant risk factor for AD (reviewed in 13-15). For example, in several recent studies elevated cholesterol was shown to increase relative risk for AD by a significant degree (16-18). Moreover, retrospective epidemiological studies demonstrated that individuals treated with statins, which are cholesterol-lowering drugs, are at decreased risk for developing AD (19, 20). Given the epidemiological data, it would be important to understand the relationship between elevated cholesterol and increased risk for AD, to determine whether therapies aimed at reducing plasma cholesterol might be beneficial in AD. One can envision several mechanisms by which cholesterol

could affect A β formation and/or A β clearance. For example, alterations in membrane lipid composition might have effects on secretases, at least three of which are transmembrane proteases (21-23). It is also possible that the cleavage of APP, itself a transmembrane protease, by secretases might depend on membrane and lipid composition surrounding APP. Finally, the localization of these proteins may be differentially affected by lipid composition thereby altering the cleavage of APP. These mechanisms would alter A β formation. At the same time, cholesterol may also affect A β clearance. For example, apolipoprotein E is found in lipid particles that include varying amounts of cholesterol and it is conceivable that altering the cholesterol content of apolipoprotein E-containing lipid particles may alter the interaction between the apolipoprotein E in these particles and A β and thereby affect A β clearance. In summary, given the important role for A β levels in the initiation and progression of AD, we began with the hypothesis that increased cholesterol may be a risk factor for AD because of effects of cholesterol on A β formation and/or on A β clearance.

We examined the effects of lovastatin, a widely used cholesterol lowering agent that can enter the CNS (24), and its active metabolite lovastatin acid, on reducing A β formation *in vitro* (25) and we examined the effects of a controlled-release formulation of lovastatin on A β levels *in vivo* (26). We observed that pharmacologically attainable concentrations of these compounds reduced A β levels.

3. MATERIALS AND METHODS

3.1. Materials

EasyTagTM EXPRESSTM Methionine Protein Labeling Mix, [³⁵S] (spec. activity >1000 Ci/mMol) was obtained from NEN Life Sciences, Boston MA. Fetal Calf Lipid Depleted Serum (FCLPDS) was obtained from Intracel, Rockville MD. Dulbecco's modified Eagles Medium (DMEM) was obtained from BioWhittaker, Walkersville MD. Dulbecco's phosphate buffered saline (PBS) and Fetal Bovine Serum (FBS) were obtained from Life Technologies, Rockville MD. Both lovastatin and lovastatin acid (LA) were freshly dissolved in dimethyl sulfoxide (DMSO). Antibody 6E10 was obtained from Senetek, Napa CA. Agarose bound antisera anti-mouse IgG was obtained from American Qualex Antibodies, San Clemente CA. Protein A sepharose was obtained from Pharmacia Biotech, Piscataway NJ. Tissue culture plates were obtained from Falcon, Lincoln Park NJ with the exception of the 10 mm culture dishes with glass coverslips that were obtained from MatTek Corporation, Ashland MA. All other chemicals were obtained from Sigma, St. Louis MO.

3.2. Cells

Chinese Hamster Ovary (CHO) cells expressing the 751 amino acid form of the amyloid protein precursor (APP) were a generous gift from Dr. E. Koo. Madin-Darby canine kidney (MDCK) cells that overexpress the 695 truncated amino acid form of APP were a generous gift from Dr. S. Sabo. H4 cells overexpressing the 695 form of

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human APP were a generous gift from Dr. W. Wasco. All cell lines were maintained in DMEM containing 10% FBS and antibiotics. To study the effects of cholesterol depletion, cells were plated onto 6 well plates in DMEM containing 10% FCLPDS with or without indicated concentrations of either lovastatin or LA for 4 days. For filipin staining, cells were plated in media containing DMEM with 10% FCLPDS and antibiotics on 10 mm plates which contained a poly-D-lysine coated coverslip.

3.3. Assay of APP processing

Cells were grown on six-well plates for 4 days in DMEM containing 10% FCLPDS in the presence or absence of lovastatin or LA. The medium was removed and the cells were washed once with PBS and then incubated for 2 hours in DMEM containing 1 mCi/ml [35 S] Methionine. After this "pulse" period, the cells were either lysed to measure total labeled APP, or the cells were incubated for 2 hours in fresh, complete medium ("chase"). At the end of this incubation, the supernatant was collected and the cells were lysed. To measure cell-associated, full-length immature and mature APP, or to measure carboxyl-terminal fragments of APP, cell lysates were incubated with antibody 369 that recognizes the carboxyl-terminus of APP (27). To measure secreted, carboxyl-terminal truncated APP (APP_s) or to measure A β , cell supernatants were incubated with antibody 6E10 (28), which recognizes the first 15 amino acids of A β (that correspond to the COOH-terminal amino acids of APP_s). The incubations with primary antibody were performed at 4°C for 75 minutes followed by a 45 minute incubation at 4°C with either agarose-linked anti-mouse IgG (for 6E10) or protein A sepharose (for 369). The beads were then washed three times for 10 minutes and then run on either a 10-20% Tris-Tricine Gel (for APP_s and A β) or an 8% polyacrylamide gel (for cell-associated APP). The gels were dried and exposed to a PhosphorImager screen and exposed for a minimum of two days. The protein bands were visualized on a STORM 860 Phosphor Imager (Molecular Dynamics) and quantitated using ImageQuant (Molecular Dynamics). To normalize for protein expression, the levels of APP and APP fragments measured after the 2 hour chase were expressed as a fraction of the amount of labeled immature APP observed after the 2 hour pulse (see 27).

3.4. Filipin staining

Cells were plated onto 10 mm culture dishes and incubated in the presence or absence of LA. Following incubation, cells were washed once with PBS and fixed with 3% paraformaldehyde in PBS for 1 hour, followed by washing 3 times in PBS for 5 minutes and quenching with 1.5 mg/ml glycine in PBS for 10 minutes. The cells were subsequently stained with 0.5 mg/ml filipin in PBS for 2 hours and washed 3 times for 5 minutes in PBS. After the final wash, the cells were visualized under a fluorescent microscope.

3.5. Study subjects

The *in vivo* study was conducted in accordance with the Declaration of Helsinki, as amended in Venice and Hong Kong in 1983 and 1989, with the Good Clinical Practices of the U.S. Code of Federal Regulations (21 CFR

50, 56, and 312), and with the Guideline for Good Clinical Practices of the International Conference on Harmonization (U.S. Federal Register May 9, 1997). The study protocol, protocol amendments, and informed consent were approved by the Institutional Review Board of each site participating in the study. All study subjects provided written informed consent prior to participating in any study related activities. Men and women aged 21 to 70 years who met National Cholesterol Education Program criteria for treatment with an HMG-CoA reductase inhibitor and did not have significant comorbid medical conditions were recruited for this study. Subjects discontinued use of all lipid-modifying agents at least 4 weeks prior to evaluation for enrollment. Concomitant medications were typical for subjects in this age group (e.g., non-steroidal anti-inflammatory agents, antihypertensives), and were kept constant during the study to the extent clinically practicable. Following enrollment, subjects were treated with single-blind placebo for 4 weeks. They were then randomly assigned to continued double-blind treatment with placebo or with 10, 20, 40 or 60 mg per day of controlled-release lovastatin (Andrx Corporation, Fort Lauderdale, FL). Serum for assay of A β concentrations was obtained weekly during the single-blind placebo run-in period, and after 4, 8, 11 and/or 12 weeks of randomized treatment, as possible. Baseline was defined as the average of the serum A β concentrations 1 week prior and just prior to the first dose of randomized treatment. Endpoint was defined as the average of A β levels at 11 or 12 weeks of treatment or the last sample obtained if an 11- or 12-week sample was not available. The mean subject age was 57 years (range 29 to 70 years).

3.6. Serum Ab assays

Serum concentrations of A β were assayed using an enzyme-linked immunosorbent assay (ELISA) as previously described (11). Briefly, 96-well plates (Falcon Probind) were coated with 4G8 monoclonal anti-A β antibody (Senetek Crude IgG Ascites Fluid) in carbonate-bicarbonate buffered solution (Sigma) and then incubated at 37°C for 12-16 hours. The plates were then washed with ECW buffer (PBS, 0.1% BSA, 0.05% Tween-20, 0.2% CHAPS, 5mM EDTA, 2mM betaine, 0.05% NaN₃) before adding ECW buffer containing 1% casein and incubated at 37°C for an additional 4 hours. The coated plates were washed with ECW before loading standards or samples. Standard curves of synthetic A β 1-40 peptide were prepared at final concentrations of 0 – 500 pg/ml. Serum samples were thawed, sonicated, and added to wells in quadruplicate. Plates were incubated 2 days at 4°C, washed, and biotinylated 6E10 monoclonal antibody (Senetek mAbs Biotin 6E10) was added to each well. After incubating at room temperature for 12-15 hours, the plates were washed, and streptavidin alkaline phosphatase (Amersham) was added to each well and incubated at room temperature for 5 hours. The plates were washed, and Attophos reagent (JBL Scientific Inc) was added to each well and allowed to develop at room temperature in the dark. Plates were read on a microplate reader (PerSeptive Biosystems CytoFluor Series 4000) at an excitation of 450 nm and an emission of 530 nm. The 4G8 antibody recognizes an epitope between residues 17 and 20 of A β , and 6E10 recognizes an epitope around residue 11 (11). As

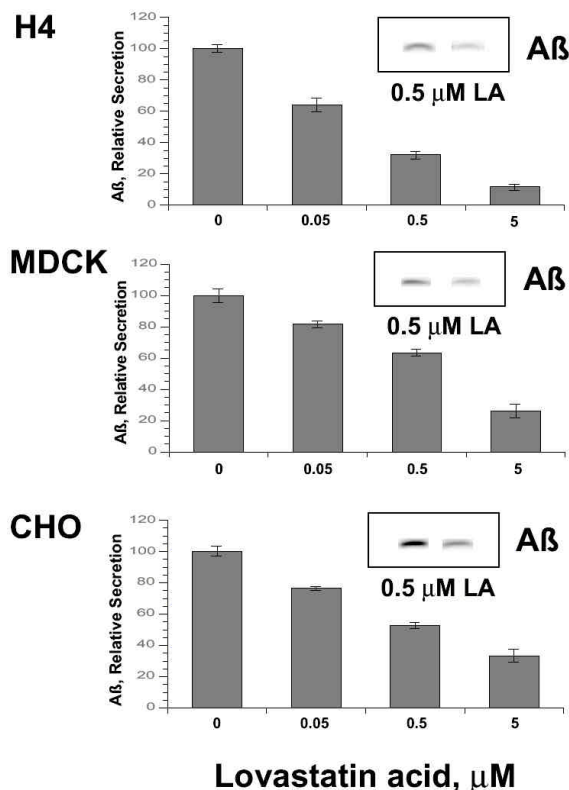


Figure 1. Effects of lovastatin acid on A β formation. Human neuroglioma (H4) cells, canine kidney (MDCK) or Chinese hamster ovary (CHO) cells were incubated for 4 days in the presence of lipid-depleted serum and in the absence or presence of the indicated concentrations of LA. At the end of this period, the cells were incubated for 2 hours in the presence of [35 S] methionine in methionine- and serum-free medium (“pulse”), followed by a 2 hour incubation in serum-free medium containing unlabeled methionine (“chase”). The indicated concentrations of LA were included in both pulse and chase. [35 S]-labeled A β was immunoprecipitated from the cell culture supernatant, resolved by SDS-PAGE, and visualized by autoradiography (insets). Relative levels of A β were determined under each condition by quantitative PhosphorImager autoradiography. Data under each condition were normalized for total [35 S]-labeled immature APP found in the cell after the 2 hour pulse to exclude any changes in A β levels due to decreased synthesis of A β . Data represent the mean \pm SEM of four experiments performed in quadruplicate.

used in the ELISA, the antibodies recognize only A β , not its precursor APP or large APP derivatives (11).

3.7. Statistical analyses

For the *in vitro* studies, data were analyzed by ANOVA followed by Fisher’s post hoc test. For the *in vivo* studies, assay values were converted to percent change from baseline. The percent change values were subjected to an analysis of variance (ANOVA) procedure employing Dunnett’s correction for comparison of each active group to the placebo group. Confirmatory analyses using ANOVA on the ranked data were also conducted.

4. RESULTS

4.1. Lovastatin acid decreases A β formation in cultured cells

In order to characterize the effects of cholesterol depletion on A β secretion, cells were cultured in fetal calf lipid depleted serum (FCLPDS) and either lovastatin or its active metabolite lovastatin acid (LA). Growing cells in lipid-depleted medium reduces the external source of cholesterol and both lovastatin and LA inhibit the synthesis of cholesterol in the cell. Filipin staining, used as a measure of the level of cholesterol in the membrane, demonstrated a decrease in cholesterol levels in cells incubated with lovastatin or LA as compared to control (25). Not surprisingly, given that LA is the functional inhibitor, LA was more effective than an identical concentration of lovastatin.

To determine whether a decrease in cholesterol is associated with a change in A β , we incubated three distinct cell types expressing human APP for 4 days in the presence or absence of 0.5 μ M LA (figure 1, insets). The cells were then incubated in media containing 1mCi/ml [35 S] Methionine for 2 hours followed by incubation in complete fresh medium for an additional 2 hours. By subsequently focusing only on the [35 S]-labeled protein, we could normalize the amounts of [35 S]-labeled A β to the levels of total immature [35 S]-labeled APP found at the end of the 2 hour chase and thereby restrict our analysis to the proteolysis of APP and secretion of A β (27). The levels of A β secretion in the presence of 0.5 μ M LA decreased by 40-60% as compared to the untreated cells (figure 1, insets). Treatment of cells with 0.5 μ M lovastatin had a weaker effect on A β levels (< 20% reduction) (data not shown).

To determine the effective range of concentrations of LA, each cell type was grown in the absence or presence of various concentrations of LA (figure 1, bargraphs). In each cell type, the level of A β secretion decreased with increasing LA concentration and a concentration of 50 nM LA or higher was sufficient to significantly ($p < 0.001$) decrease the level of A β secretion under these experimental conditions (figure 1). There was no evidence of toxicity under these conditions, an important consideration, given that toxicity could lead to an apparent decrease in A β production.

Newly synthesized (“immature”) APP undergoes glycosylation and sulfation in the Golgi apparatus, resulting in a “mature” APP that can then be exported from the trans-Golgi network and cleaved and processed (figure 2, upper panel). α -Secretases (22, 23) cleave mature APP within the A β domain and release APP_s into the extracellular space. β -Secretase(s) (21) cleave at the NH₂-terminal of the A β domain and, when this activity is followed by γ -secretase activity, A β is formed. The A β thus formed is typically released from the cell, although it may also accumulate within the cell. To determine whether the decreased levels of A β we observed were due to decreased formation, rather than decreased release, we measured the levels of [35 S]-

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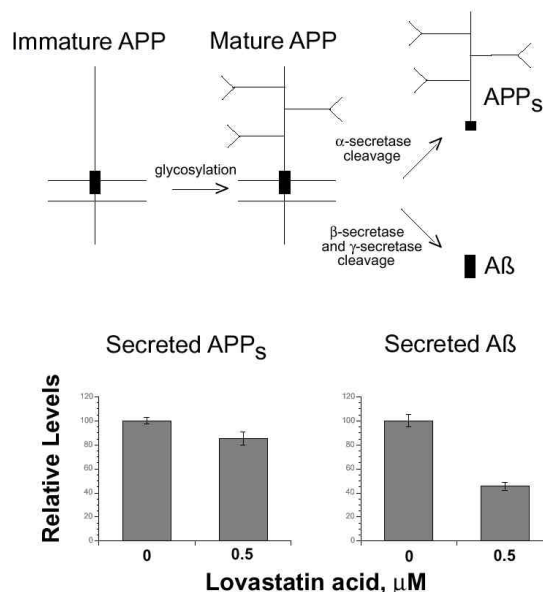


Figure 2. Effects of lovastatin acid on APP processing. *Upper.* New synthesized (“immature”) APP undergoes glycosylation and sulfation to produce the mature species. Mature APP can be cleaved by α -secretases to release APP_s, or can be cleaved by β - and γ -secretases to release A β . *Lower.* Human neuroglioma (H4) cells were incubated for four days in the presence or absence of 0.5 μ M LA, and subjected to metabolic labeling as described in the legend to figure 1. The levels of [35 S]-labeled immature APP and mature APP were then determined by immunoprecipitation of these species from cell lysates with an antibody against the COOH-terminal of APP, followed by quantitative autoradiography. Similarly, the levels of [35 S]-labeled APP_s and extracellular and intracellular A β were determined by immunoprecipitation of these species from either cell culture supernatants (extracellular A β) or cell lysates (intracellular A β). The amounts of each species were normalized to the levels of [35 S]-labeled immature APP found in cells at the beginning of the chase. Data represent the mean \pm SEM of four experiments performed in quadruplicate.

labeled intracellular A β within the cell. We did not observe detectable levels of intracellular A β in cells incubated in the presence or absence of 0.5 μ M LA (data not shown). Therefore, the decrease in extracellular A β could not be explained by decreased secretion of A β and represents decreased formation of A β from APP.

We also determined whether cholesterol treatment affects other aspects of APP processing. We observed a modest decrease in [35 S]-labeled APP_s formation in cells incubated in the presence of LA, as compared to control cells (figure 2, lower panel). Because mature APP is likely to be the precursor for both APP_s and A β , decreased formation of both APP_s and A β might suggest a decrease in the levels of mature APP. To examine this, we measured the levels of [35 S]-labeled mature APP in cells incubated in the absence or presence of 0.5 μ M LA. We observed no significant effect of LA on the maturation

of APP (data not shown). Therefore the effects of LA on A β levels could not be accounted for by decreased maturation of APP and instead reflect effects of LA on the post-Golgi processing and/or trafficking of APP.

4.2. Lovastatin decreases Ab levels in patients

We then assessed the effects of treatment with a controlled-release formulation of lovastatin on serum levels of A β in individuals (aged 21 to 70 years) who met National Cholesterol Education Program criteria for treatment with an HMG-CoA reductase inhibitor and did not have significant comorbid medical conditions. Of the 172 subjects who were randomized, 160 completed the study, and usable samples for A β analyses were obtained from 94 (46 males and 48 females). As expected, there were dose dependent and statistically significant decreases in low-density lipoprotein cholesterol and related lipid parameters (data not shown). The results of the serum A β assays are summarized in figure 3. The mean serum A β concentration prior to treatment was 289 ± 25 (SEM) pg/ml, and an ANOVA indicated that there were no statistically significant differences among dose groups at baseline. Mean changes from baseline in serum A β showed a dose dependent decrease, and were $+37.4 \pm 30.2$ % for placebo, $+4.1 \pm 12.6$ % for the 10 mg group, $+0.6 \pm 10.2$ % for the 20 mg group, -23.6 ± 10.7 % for the 40 mg group, and -21.3 ± 10.3 % for the 60 mg group. Median changes from baseline also showed a dose dependent decrease, and were -1.1 % for placebo, -5.9 % for the 10 mg group, -14.0 % for the 20 mg group, -38.6 % for the 40 mg group, and -31.7 % for the 60 mg group. ANOVA indicated a statistically significant effect of treatment with controlled-release lovastatin ($p \leq 0.0348$). The differences between the 40- and 60-mg groups and the placebo group were statistically significant (Dunnett’s $p < 0.05$). This result was confirmed when ANCOVA was conducted using baseline A β as a covariate. ANOVA on the ranked values for A β change from baseline was conducted to further test the response, since differences were noted between the mean and median values. This analysis also indicated a statistically significant treatment effect ($p \leq 0.0076$), with statistically significant differences between the 40- and 60-mg groups and the placebo group (Dunnett’s $p < 0.05$).

5. DISCUSSION

5.1. The central role for Ab in the biology of AD

The profound accumulation of A β in the brain as amyloid in plaques is a hallmark of AD (reviewed in 29). Amyloid load, as measured by volume of tissue occupied by amyloid, and total A β levels, as measure by ELISA, correlate inversely with cognitive levels as measured by the clinical dementia rating (CDR) (9-12). Increase in amyloid load and total A β are observed at the earliest signs of cognitive change (CDR 0.5-1) (9-12) and can precede Alzheimer-associated neuritic changes (11). These observations implicate A β both in disease initiation and the cognitive decline observed throughout disease progression.

Genetic analyses also point to A β formation and accumulation as precipitating factors in AD (reviewed in

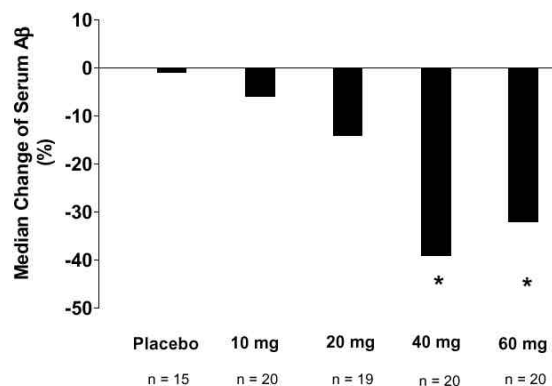


Figure 3. Effects of controlled-release lovastatin on human serum β -amyloid concentrations. Men and women aged 21 to 70 years who met National Cholesterol Education Program criteria for treatment with an HMG-CoA reductase inhibitor were enrolled in the study. Following single-blind treatment with placebo for 4 weeks, subjects were randomly assigned to double-blind treatment with the indicated doses of controlled-release lovastatin or placebo for up to 12 weeks. Serum A β concentrations were measured by ELISA and solid bars indicate median values for percent change from baseline to endpoint for the indicated dose groups. *, $P < 0.05$ by Dunnett's test; n, number of subjects per group.

3). Very rare mutations in APP that lead to early-onset, familial AD all increase A β production in both cultured cells and experimental animals. Expressing these mutations in transgenic mice leads to the development of amyloid plaques in these animals; amyloid plaques are never seen in control animals (reviewed in 29). Furthermore, these plaques can be surrounded by dystrophic neurites with abnormally phosphorylated tau, similar to neuritic plaques in AD. More common – but still quite rare – mutations in the presenilins also lead to early-onset familial AD. Expressing mutant presenilin in cultured cells or in transgenic animals leads to increased formation of A β . Expressing presenilin mutations in transgenic mice already expressing mutant human APP accelerates the formation of amyloid plaques, when compared to animals expressing the mutant human APP with no exogenous presenilin. A significant genetic contribution to AD risk is the presence of the E4 variant of Apolipoprotein E (reviewed in 3). Apolipoprotein E4 (Apo E4) is a variant that occurs in approximately 15% of the human population and increases risk for AD by ~3-fold. The means by which Apo E contributes to AD is still controversial, however, it had been known that Apo E complexes with A β (4-7). Recently, it was demonstrated that introducing human Apo E into mice led to a plaque formation in an allele-dependent fashion (8). Therefore, the genetic analysis of AD, like the postmortem studies outlined above, strongly support a definitive role for A β formation and accumulation in the initiation and progression of AD. Therapeutic interventions that reduce the formation and/or accumulation of A β would therefore be expected to delay the onset and progression of AD.

5.2. Summary of the findings from our studies

In the studies presented above, we asked whether lovastatin and lovastatin acid might alter the formation of A β , in addition to decreasing cholesterol formation. The rationale for such studies is the observation that hypercholesterolemia is a significant risk factor for AD (16-18) and statins may be protective against AD (19, 20). In our *in vitro* studies, we incubated cells with medium containing lipid-depleted serum, rather than normal, lipid-rich serum. This decreased the exogenous pool of cholesterol available to the cell. Typically such a reduction in available cholesterol would induce cholesterol synthesis by the cell. To block cholesterol synthesis, cells were incubated in lovastatin acid (LA), which blocks this process. Under the condition of our study, we found that LA significantly reduced A β formation in 3 distinct cell types concomitant with a reduction in cholesterol. We tested LA at concentration of 0.05-5 μ M and observed decreases in A β formation even at the lowest of these doses. The effects of LA on A β formation show some specificity, because the secretion of COOH- terminally truncated, secreted APP (APP_s) was not affected to the same degree. The minimal effect of LA on APP maturation and APP_s formation would exclude toxic effects of LA as a cause for decreased A β formation. In addition, the data suggest that LA does not affect the normal, presumably beneficial maturation and processing of APP.

For the *in vivo* studies, both median and mean values for percent change from baseline across treatment groups indicated greater reductions in A β levels with increasing doses of controlled-release lovastatin. Within groups, differences were observed between mean and median values. For example, the mean change from baseline in the placebo group (+ 37.4%) was notably different from the median change from baseline in the placebo group (-1.1%). The differences between means and medians suggest that the values were not normally distributed, with a small number of placebo-treated cases showing large increases from baseline. The increase of mean values for the placebo group, while not statistically significant, could reflect effects of cessation of cholesterol-lowering drugs by patients before enrollment in the current study. Furthermore, a floor effect may have contributed to the distributions, since percent change from baseline values following treatment could increase by greater than 100%, but could not decrease by more than 100%. To address these issues we looked at median values and used a non-parametric procedure (ANOVA on the ranked data), which indicated that the treatment effects were significant. Thus, the results from this study indicated that treatment with controlled-release lovastatin can lower serum levels of A β .

5.3. Other studies on cholesterol lowering agents, cholesterol and Ab

There have been additional studies looking at cholesterol and A β formation in cultured cells and in animals. Studies in cultured cells made use of several methods to modulate cholesterol levels, and it is important to consider these methods when developing a hypothesis for the mechanism by which reducing cholesterol may

modulate A β formation. A common approach for depleting cholesterol has been to culture cells with or without a statin, followed by a brief exposure to the cyclodextrin methyl- β -cyclodextrin (MBC). Cyclodextrins are compound that strips cholesterol from the outer leaf of the cell plasma membrane. Unfortunately, while MBC has very rapid effects on plasma membrane cholesterol, it may have little effect on cholesterol levels within the cell. Furthermore, in our studies, MBC proved toxic to several different cell types as evidence by cell death in exposures over 60 minutes. The alternative approach, which we used here, is to culture cells in the presence of a statin, without the use of a cyclodextrin. The viability of cells under these conditions remains high. To increase cholesterol levels, exogenous cholesterol can be added. Finally, one can also modulate the levels of cholesterol-esters, as noted below.

With regards to studies using MBC in cultured cells, the first report demonstrated that A β formation, but not APP_s formation, was decreased in treated hippocampal neurons (30). Subsequently, it was shown that depleting cholesterol in this manner can increase APP_s formation in human embryonic kidney cells (HEK 293), human astrogloma (U373), human neuroglioma (H4), and human neuroblastoma (SH-SY5Y) cells (31). Finally, a recent study showed that use of simvastatin or lovastatin with MBC reduced both A β 40 and A β 42 formation in primary neuronal cultures (32).

When cells are incubated with lovastatin or lovastatin acid alone, without the use of MBC, A β levels are decreased (without an increase in APP_s) and the decreases in A β are due to the decreased conversion of APP to A β (current findings, 25).

Addition of cholesterol (solubilized with MBC or ethanol) to HEK 293 cells decreased APP_s formation and slowed APP maturation (33). Addition of cholesterol solubilized in MBC to HEK 293 cells increased A β 40 and A β 42 formation (34). In primary neuron and glial cultures, addition of MBC-solubilized cholesterol modified the glycosylation of APP and led to decreased APP_s formation (35). Finally, it has been shown that the equilibrium of cholesterol and cholesterol esters in cultured cells may be a determinant of A β formation (36). These researchers demonstrated that inhibiting the enzymes that catalyzes the formation of cholesterol esters leads to a decrease in A β levels.

Rabbits fed with a cholesterol-rich diet demonstrated increased A β (and Alz-50) immunoreactivity, although A β levels were not directly assessed (37). In one study, similarly fed mice expressing mutant human APP had decreased levels of secreted APP_s and decreased levels of A β 40 and A β 42 (38). However, in more recent studies in mice a high cholesterol diet led to increases in deposited A β and decreases in APP_s, which correlated with the levels of plasma and total CNS cholesterol (39). Treating such mice with BM15.766, an inhibitor of 7-dehydrocholesterol-D7-reductase, decreased deposited A β (40). Similarly, treating guinea pigs with the HMG-CoA reductase inhibitor

simvastatin led to reduced CSF and brain levels of total A β , A β 40, and A β 42 (32).

5.4. Potential mechanisms for the effects of cholesterol lowering agents on A β

We have outlined in the Introduction some mechanisms by which cholesterol levels may regulate A β levels by altering its formation, by acting on the localization or activity of APP and/or the enzymes that cleave APP, or its clearance, by acting on Apo E particles. The studies described above support a role for cholesterol in A β formation *in vitro*, and by extension *in vivo*. There have been several studies aimed at addressing how cholesterol might regulate A β formation. One potential mechanism is that cholesterol depletion may alter the appropriate biosynthesis of APP, the precursor to A β (35). A more recent study suggests that cholesterol effects on A β are due to the effects on cholesterol on production of secreted APP_s (31). The authors suggest that decreasing cholesterol leads to stimulation of the α -secretase ADAM 10. It should be noted, however, that the effects of reduced cholesterol on APP_s were not seen in several studies, including our own, nor did we see any evidence for altered biosynthesis of APP. A related hypothesis is that cholesterol depletion alters the distribution of the β -secretase, BACE (41). These researchers show that BACE, normally localized to lipid rafts along with APP, is redistributed when cholesterol is depleted using MBC.

Another question to bear in mind is whether it is in fact cholesterol *per se* that is important in regulating A β formation. The studies with cholesterol esters may suggest that it is not total cholesterol that is important but rather cholesterol esters (36). Finally, in one epidemiological study it was shown that statins, but not other cholesterol lowering drugs, were apparently protective against AD (20). This has been the basis of the suggestion that it is a property of statins not related to cholesterol effects that are important for the protective effects of these compounds (see 13).

5.5. Future directions

Our placebo-controlled clinical study represents the first such published study demonstrating an effect of a statin on A β in humans. Based on our prior work with lovastatin acid in cultured cells, and on the related studies summarized above, we argue that there is likely to be a direct effect of lovastatin acid on A β formation *in vivo*. If lovastatin or related compounds decrease A β *in vivo*, they may be therapeutic in early AD and decrease risk for AD.

Several questions remain. First, will statins, typically targeted to the liver, be able to be effective in a CNS disorder. Certain statins, including lovastatin, can enter the CNS (24). This property might be augmented in formulations that increase the circulating levels of the statin (as observed with controlled-release formulations). Furthermore, A β can be transported through the blood-brain barrier by active transport mechanisms (42-44). Therefore, A β in the periphery (i.e., in the blood) may pass into the central nervous system (CNS) and vice versa. If

this is the case, decreasing the levels of A β in the periphery may lead to reduced A β levels in the CNS. In a dramatic example of the latter possibility, it has recently been shown that inducing antibodies to A β in transgenic mice expressing mutant human APP led to decreased A β levels in the blood and profound decrease in amyloid plaque levels in the brain of these animals (45). This has been demonstrated to be the result of a re-partitioning of A β from the CNS to the periphery, demonstrating that decreasing peripheral A β will lead to reduced A β in the CNS (46).

A second question is whether A β levels in plasma, serum or CSF can be used to estimate efficacy of an anti-Alzheimer drug. We believe the evidence argues for a central, causative role for A β in AD and we therefore consider that decreasing A β will slow progression. However, a prospective study of statins looking at cognitive measure in patients or people at risk will ultimately be necessary. Such a study might be simplified if one were to determine the doses of the statin in the trial by using a shorter-term trial looking at A β levels.

We would also like to underscore that it is by no means certain that it is the cholesterol-lowering properties of statins that lead to reduced A β and apparent protection. As noted above, in one epidemiological study certain classes of cholesterol-lowering drugs were ineffective at protecting against AD (20). For this reason, it would be a grave mistake to generalize from one class of drugs to other classes of drugs with similar indications, and it would even be a grave mistake to generalize from one drug within a class to other drugs within that class. In this regard, we again would argue that A β measures may be a rational first pass to evaluate specific drugs for potential Alzheimer trials.

In summary, epidemiological studies suggest that statins may be beneficial in delaying the onset and/or slowing the progression of AD. In vitro studies with statins, including lovastatin, indicate that these compounds decrease A β formation *in vitro* and *in vivo*, suggesting a mechanism by which such compounds may be protective. Further study of such compounds in humans for clinical efficacy is therefore indicated. We demonstrate that a controlled-release formulation of lovastatin reduces A β levels in clinical trials, which may support its efficacy as an agent for delaying the onset and/or slowing the progression of AD.

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Abbreviations: A β : β -amyloid peptide, AD: Alzheimer's disease, Apo: apolipoprotein, APP: amyloid protein precursor, APP_s: secreted, COOH-terminally truncated APP, CDR: clinical dementia rating, ELISA: enzyme-linked immunosorbent assay, FCLPDS, fetal calf lipid depleted serum, HMG-CoA: hydroxymethylglutaryl-coenzyme A, MCB: methyl- β -cyclodextrin

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