Lovastatin Differentially Affects Neuronal Cholesterol and Amyloid- β Production *in vivo* and *in vitro*

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SUMMARY

Background and Aims: Epidemiological and experimental studies indicate that high cholesterol may increase susceptibility to age-associated neurodegenerative disorders, such as Alzheimer's disease (AD). Thus, it has been suggested that statins, which are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), may be a useful therapeutic tool to diminish the risk of AD. However, several studies that analyzed the therapeutic benefits of statins have yielded conflicting results. Herein, we investigated the role of lovastatin on neuronal cholesterol homeostasis and its effects on amyloid β protein production *in vivo* and *in vitro*. **Methods and Results:** Lovastatin effects were analyzed *in vitro* using differentiated human neuroblastoma cells and *in vivo* in a lovastatin-fed rat model. We demonstrated that lovastatin can differentially affect the expression of APP and A β production *in vivo* and *in vitro*. Lovastatin-induced HMGCR inhibition was detrimental to neuronal survival *in vitro* via a mechanism unrelated to the reduction of cholesterol. We found that *in vivo*, dietary cholesterol was associated with increased A β production in the cerebral cortex, and lovastatin was not able to reduce cholesterol levels. However, lovastatin induced a remarkable increase in the mature form of the sterol regulatory element-binding protein-2 (SREBP-2) as well as its target gene HMGCR, in both neuronal cells and in the brain. **Conclusions:** Lovastatin modifies the mevalonate pathway without affecting cholesterol levels *in vivo* and is able to reduce A β levels only *in vitro*.

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Introduction

Epidemiological and experimental studies have linked changes in cholesterol metabolism to an increased susceptibility to AD [1–3]. In fact, the $\epsilon 4$ allele of ApoE, which is associated with high cholesterol, is a well-established risk factor for AD [4,5]. A β deposition in the brain is considered an important factor in AD progression, as it initiates a cascade of events associated with synaptic damage and neuronal death [6]. The majority of APP is constitutively cleaved within the A β sequence by α -secretase enzymes, preventing A β production. In the amyloidogenic pathway, APP is proteolyzed by β -secretase, which is further cleaved by γ -secretase, leading to A β formation [7–9]. High-cholesterol intake has been linked to the accumulation of A β in the brain, suggesting that high cholesterol might increase the amyloidogenic processing of APP and that, in contrast, low cholesterol favors the nonamyloidogenic pathway, decreasing A β production and amyloid plaque formation [10–12]. Changes in membrane cholesterol levels or in its distribution in lipid rafts have been suggested to influence the activity and expression of the enzymes involved in the amyloidogenic processing of APP [10,11,13,14]. We have previously shown

that neuronal exposure to high cholesterol worsens A β toxicity by increasing the formation of ROS [15]. Thus, cholesterol may increase the brain's vulnerability to A β at two levels: by promoting A β deposition and by increasing its toxicity. Therefore, it has been suggested that statins, which are pharmacological inhibitors of HMGCR [16], may be useful in diminishing the risk of AD [17–19]. While some reports have proposed the use of statins due to their cholesterol-lowering properties and their ability to decrease A β formation [13,20], their beneficial effects remain controversial [21–23]. Statins have many positive effects in the CNS, including effects on cell survival [24,25] and neuronal plasticity [26,27]. Statins also display anti-amyloidogenic activity [13,28–31] and antioxidant [32,33] and anti-inflammatory properties [34,35]. In contrast, statin-mediated neurotoxicity has also been reported [36–42], including the inhibition of neurite outgrowth [43–45] and the impairment of neurotransmission [46,47]. In addition to their cholesterol-lowering properties, statins have pleiotropic actions related to inhibition of the mevalonate pathway, which lowers the concentrations of biologically active isoprenoid intermediates, such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP).

Circulating cholesterol, however, is regulated differently than cholesterol in the brain (revised by Mendoza-Oliva et al. [48]). Due to existing controversy about the relationship between cholesterol and AD [49], it is important to understand how cholesterol in the brain is regulated and how this process is affected by statins before we can determine their potential and safety for use in treating AD. The aim of this study was to explore the effects of lovastatin, a lipophilic statin able to cross the blood–brain barrier [50], on cholesterol homeostasis and neuronal survival. We also aimed to uncover any links with APP expression and A β metabolism using a human neuroblastoma cell model and an *in vivo* lovastatin-fed rat model.

Materials and Methods

Cell Culture

Human neuroblastoma cells (MSN) [51] were maintained in RPMI 1640 (Gibco Invitrogen, Grand Island, CA, USA) medium containing nonessential amino acids plus 10% fetal calf serum (Gibco Invitrogen). Cells were grown in petri dishes (100 $\times$ 15 mm) or in 24-well plates at a density of 1×10^6 cells per well in an atmosphere of 5% CO₂ /95% O₂ at 37°C. After 24 h, MSN cells were differentiated by the addition of retinoic acid (10 μ M) and nerve growth factor (NGF) (50 ng/mL), and cells were exposed to different concentrations of lovastatin (2 or 10 μ M; Calbiochem, Temecula, CA, USA) or cholesterol (25 μ M; Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol (0.1 and 0.16%, respectively) and cultured in normal (10%) or serum-free media for an additional 2 or 5 days. In some experiments, cholesterol and lovastatin were added simultaneously during 2 or 5 days.

Cell Viability

Cell viability was assessed through the conversion of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) to formazan crystals by mitochondrial respiratory chain reactions [52]. The reduction of MTT is an indicator of mitochondrial redox capacity that is used as a measure of cell viability. In brief, MTT in phosphate-buffered saline (PBS) (5 mg/mL) was added to MSN cells for 1 h at 37°C in a 1:10 (v/v) ratio after incubation with lovastatin. The incubation medium was removed, and neurons were solubilized with isopropyl alcohol (0.8 mL). The absorbance of each sample was quantified using a spectrophotometer at 570 nm (Pharmacia Biotech, Piscataway, NJ, USA).

Cholesterol Measurement

The total cholesterol content of MSN cells or brain samples was quantified using the Amplex Red cholesterol assay (Molecular Probes, Eugene, OR, USA). Briefly, cells or tissues were homogenized in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% desoxicolate and protease inhibitors diluted in reaction buffer. An equivalent volume of Amplex Red working solution (300 μ M Amplex Red, 2 U/mL cholesterol oxidase, 2 U/mL cholesterol esterase

and 2 U/mL horseradish peroxidase) was added. The samples were incubated at 37°C for 30 min in the dark, and the absorbance was measured at 535 nm using a spectrophotometer (microplate reader, model 550; Bio-Rad, Hercules, CA, USA). The cholesterol content was calculated by interpolating from a standard curve of cholesterol and then normalized with respect to the protein content from each sample. The concentration of cholesterol in the culture media was approximately 132.33 μ M; for this reason, we had to remove the serum from the media to analyze the cholesterol-lowering effect of lovastatin.

Cholesterol Labeling

MSN cells were grown on coverslips, washed twice in PBS for 5 min, and stained with filipin (Sigma-Aldrich). This blue fluorescent probe was used to study the distribution of nonesterified cholesterol [53]. The filipin stock solution (1 mM) was prepared in methanol. A working solution (20 mg/mL) was prepared by dissolving the stock solution in PBS. The working solution was applied to cells for 45 min at 37°C. MSN cells were then exposed to lovastatin (10 or 2 μ M) for 5 days in normal and serum-free conditions. Next, the cells were washed twice with PBS for 5 min and fixed in 4% paraformaldehyde for 20 min. The fixed cells were permeabilized with PBS/Triton 0.25% for 20 min, washed with PBS and then incubated with 500 μ L of the filipin working solution. The cells were then washed three times in PBS and observed at 60 $\times$ magnification on an inverted fluorescent microscope (Olympus IX71 375) using 360 nm excitation and collecting emissions at 460 nm.

Uptake of BODIPY-FL-LDL

To analyze the incorporation of LDL into cells, we used fluorescently labeled LDL. Cells were grown on coverslips in serum-free medium and treated in the presence or absence of lovastatin (2 μ M) for 12 h. Next, cells were incubated in fresh media containing 10 μ g/mL of BODIPY-FL-LDL (Molecular Probes). After incubation at 37°C for 5 h, cells were washed with PBS and fixed in 1% paraformaldehyde. The uptake of BODIPY-FL-LDL (Ab 515 nm, Em 520 nm) was detected using a DSU confocal microscope at 60 $\times$. Consecutive z-stacks (7–9) were flattened into a single image using the ImageJ software (NIH, Bethesda, MD, USA).

Immunohistochemistry

Cells were grown on coverslips, washed twice with PBS for 5 min, fixed with 4% paraformaldehyde for 20 min and washed again with PBS. Cells were permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature and left in blocking solution (PBS /BSA 5%) overnight at 4°C. These cells were incubated for 24 h at 4°C with mouse monoclonal anti-HMGCR (1:100; Santa Cruz Biotechnology, Dallas, TX, USA) and washed three times for 5 min with PBS. Cells were then incubated in the dark with a secondary antibody Alexa Fluor-546 (1:300; Molecular Probes) for 2 h at room temperature. Cells were also incubated with phalloidin for

20 min after which they were washed three times with PBS. Finally, cells were analyzed at 100 $\times$ magnification. Images were obtained on an epifluorescence microscope (Zeiss Axioskop 40; Oberkochen, Germany). Negative controls were prepared by omitting the primary antibody.

Quantitative Real-time Reverse Transcription-PCR

The real-time quantitative reverse transcription (qRT-PCR) was used to quantitate mRNA levels for HMGCR (human), using β -actin mRNA (human) to normalize the data. From 1 μ g of RNA, cDNA was synthesized using the reverse transcriptase enzyme M-MLV (Invitrogen, Carlsbad, CA, USA). With a few modifications, the cDNA was synthesized according to manufacturer's protocol: 150 ng/ μ L of random oligonucleotides, 7.5 ng/ μ L of oligonucleotide dT20, 10 mM of dNTPs, 0.1 M DTT and 100 units of M-MLV were used in a final volume of 20 μ L. The qRT-PCR amplification was performed on a Rotor-gene Q (Qiagen, Valencia, CA, USA) using the SYBR Greener qPCR SuperMix kit (Invitrogen). Each primer was at 200 nM in a final volume of 20 μ L per reaction. The reaction parameters were as follows: incubation at 50°C for 2 min, activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, alignment with oligos for HMGCR and β -actin at 60°C for 60 s, and a final elongation at 72°C for 2 min. Specificity of the amplified products was checked by analysis of melting curves.

The oligonucleotides used for human HMGCR were as follows: F 5'-TACCATGTCAGGGGTACGTC-3' and 5'-CAAGCCTAGAGACATAATCATC R-3' (247 bp). Oligonucleotides used for human β -actin were as follows: 5'-TGAAGGTGACAGCAGTCGGTTG F-3' and 5'-R GGCTTTTAGGATGGCAAGGGAC-3' (146 bp). These were all synthesized by Sigma. Samples were run in triplicate, and the data were analyzed using the method described by Pfaffl, 2001 [54].

In vivo Model of Lovastatin-fed Rats

Male Sprague Dawley rats weighing 250 g (Harlan Laboratories, Facultad de Química, UNAM, México) were used in this study. All of the animals were maintained under a reverse light/dark cycle and a controlled room temperature (22°C) with free access to a standard rodent diet (Harlan Laboratories, Indianapolis, IN, USA) and tap water before experimentation. All animals were handled in accordance with local government rules, the Society for Neuroscience Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Instituto de Investigaciones Biomédicas, UNAM. All efforts were made to minimize the suffering of animals and to reduce the number used.

The animals were divided into three groups: a control group, in which rats were fed a standard diet, and 2 experimental groups. In one experimental group, the animals were fed a high-fat diet (HFD) containing 1% cholesterol (Bio-Serv, Flemington, NJ, USA). The second experimental group was fed 0.04% lovastatin added to a standard diet per the instructions of Ness et al. [55] Rats were maintained on one of these three diets, with free access to water, for 5 days. After the treatment, all rats were decapitated during 4th hour of the dark cycle. Samples from the liver and the

brain (including the cortex, hippocampus, and cerebellum) were quickly dissected. The fresh samples were dipped into PBS with protease inhibitors (Completem, Roche, Basel SUI) and then stored at -70°C until analysis.

Measurement of A β

A $\beta_{[1-42]}$ and A $\beta_{[1-40]}$ levels in MSN cells were determined using an A β -specific ELISA kit (Invitrogen). After being treated as previously described, the MSN cell culture medium was collected and analyzed. The A β concentration was determined by measuring optical densities at 450 nm on a spectrophotometer (microplate reader, model 550; Bio-Rad).

The A β concentration in brain samples was determined using the same method as above, once protein was extracted from the tissue according to the manufacturer's recommendations (A $\beta_{[1-40]}$ ELISA; Invitrogen). Tissues were weighed and homogenized in an 8 $\times$ mass of cold 5 M guanidine HCl/50 mM Tris-HCl buffer and then mixed at room temperature for 3 h. The homogenized samples were diluted (1:10) with cold BSAT-DPBS buffer (Dulbecco's phosphate-buffered saline with 5% BSA and 0.03% Tween-20 supplemented with protease inhibitors) and centrifuged at 16,000 g for 20 min at 4°C. The supernatant was kept on ice and used to quantitate A β levels. All results were normalized by tissue mass and expressed as a level relative to the control values.

Immunoblotting

After experimental treatments, MSN cells or tissues were homogenized in lysis buffer. Protein samples were loaded onto a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membranes were blocked in PBS with 5% nonfat dry milk and then incubated for 2 h at room temperature. Next, the membranes were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal antibody anti-HMGCR (1:100; Santa Cruz Biotechnology), mouse monoclonal antibody anti-SREBP-2 (1:200; Chemicon, Temecula, CA, USA), mouse monoclonal antibody anti-Alzheimer precursor protein A4 (22C11) (1:1000; Chemicon), and mouse monoclonal anti- β -actin (1:1000; Sigma-Aldrich). Membranes were washed three times for 5 min each with PBS/0.1% Tween-20 and then incubated at room temperature for 2 h with a horseradish peroxidase-conjugated secondary antibody: goat anti-mouse IgG (1:10,000–50,000; Santa Cruz Biotechnology). Membranes were washed three times (5–10 min each) with PBS/0.1% Tween 20 and the antibody signal was detected by chemiluminescence (ECL kit; EMD Millipore, Temecula, MA, USA) on Kodak film. Densitometric analysis of the immunoblot was carried out using ImageJ software (NIH). The results are expressed as arbitrary optic density units (ODU).

Statistical Analysis

Data are presented as means $\pm$ SEM. Statistical significance was determined by Student's t-test. We used GraphPad Prism 6.0 (GraphPad Software, Bethesda, MD, CA, USA) for graphs and statistical analysis.

Figure 1 Effects of lovastatin on cholesterol levels and cell viability. **(A)** Cholesterol content in MSN cells quantified by the Amplex Red cholesterol assay in whole-cell lysates of neurons treated with 10 μ M lovastatin (Lov) in medium with 10% serum (dark column) or 2 μ M Lov in serum-free conditions (gray column) for 5 days. Changes in cholesterol levels are expressed as the percent change compared to control cells incubated with 10% serum or serum free in the absence of Lov (dotted lines). Each bar represents the mean $\pm$ SEM of triplicate determinations of five to six experiments. **(B)** Fluorescent images and fluorescence intensity (arbitrary units) of filipin staining in differentiated MSN cells exposed to Lov (10 or 2 μ M) for 5 days in medium with 10% serum (left panel) and without serum (right panel) show the reduced cholesterol contents when Lov was added in serum free condition at 2 μ M. Images (60 $\times$) were analyzed using the ImageJ software. **(C)** The effect of Lov on LDL uptake in serum-free medium. MSN cells were treated in the absence (Ctrl) or presence of Lov (2 μ M) for 12 hours and incubated for an additional 5 hours at 37°C in the absence (Ctrl) or presence of Lov (2 μ M) in media containing 10 μ g/mL BODIPY-FL-LDL. LDL uptake was detected by confocal fluorescence microscopy at 60 $\times$ magnification. Consecutive z-stacks (7-9) were flattened into a single image with bright field using the ImageJ software. **(D)** Cell viability at different concentrations of Lov. Cells were treated for 5 days with different concentrations (0.5–10 μ M) of Lov (gray circles) in serum-free conditions. Each point represents the mean $\pm$ SEM of three experiments performed in triplicate and is expressed as percent change with respect to control (dotted line). **(E)** The effects of Lov (10 or 2 μ M) on mitochondrial redox activity in the absence or presence of cholesterol (25 μ M) in medium with 10% serum (dark columns) and serum free (gray columns) at 2 or 5 days. The data were expressed as the percent of Ctrl values of each condition (dotted line). The mean $\pm$ SEM of three to four different experiments performed in triplicate is plotted. * $P < 0.05$ vs. Ctrl, ** $P < 0.01$ vs. Ctrl, a, b, c $P < 0.05$ vs. Lov (2 or 10 μ M).

Results

Effect of Lovastatin on Cholesterol Levels and Neuronal Survival

To evaluate the effect of lovastatin on neuronal cholesterol levels, differentiated MSN cells were exposed to lovastatin for 24 h (data not shown), 48 h, and 5 days in the presence of 10% serum and in serum-free conditions to avoid the presence of cholesterol in the media. Only after 5 days in the absence of serum was lovastatin (2 μ M) able to reduce cellular cholesterol by approximately 40%, as measured by an enzymatic assay (Figure 1A) and visualized by histochemical filipin staining (Figure 1B). In the presence of 10% serum, lovastatin did not decrease cellular cholesterol levels (Figure 1A, B) because the neurons were able to take lipoproteins from the serum. This can be observed in Figure 1C, where cells were incubated with fluorescently labeled LDL (BODIPY-FL-LDL). In the serum-free condition, lovastatin-induced cell death was potentiated in a dose-dependent manner (Figure 1D). However, as shown in Figure 1E, the extent of lovastatin-dependent neuronal death for 10 μ M lovastatin in media with 10% serum was similar to the death for 2 μ M lovastatin in the serum-free condition. To determine whether the neurotoxicity was dependent on lovastatin-induced lowering of cholesterol, we examined neurotoxicity in the presence of 10% serum and with added cholesterol (25 μ M) in the serum-free condition. However, in any case, cell death was prevented by adding cholesterol (Figure 1E), suggesting that the toxicity might be associated with an additional pleiotropic mechanism mediated by the inhibition of downstream cholesterol metabolites produced as a result of HMGCR inhibition.

Lovastatin Induces Upregulation of HMGCR in MSN Cells

Next, we determined whether lovastatin affects the expression of neuronal HMGCR, the enzyme target of its inhibition, as has been observed in liver [55]. Two HMGCR bands were identified in MSN cells, which correspond to the monomeric (~97 kDa) and the degraded (~55 kDa) forms. The degradation of HMGCR is a long-term regulatory mechanism that is influenced not only by the concentration of sterols but also by the oligomeric state of the enzyme [56]. Control MSN cells express HMGCR at very low levels, but lovastatin exposure resulted in a more than 3-fold increase of HMGCR protein after 2 and 5 days of treatment. This increase corresponded to the degraded form in particular (Figure 2A, B). A 2-fold increase in HMGCR mRNA was also observed (Figure 2C). The effects of lovastatin were also accompanied by an increase of the mature form (~50–68 kDa) of the transcription factor SREBP-2 (Figure 2A). These results, which are similar to those reported in studies of the liver after statin exposure [55], suggest that HMGCR is a target of lovastatin-mediated inhibition in neurons. Furthermore, its regulation may depend on nonsterol products derived from the cholesterol pathway because an increase in HMGCR was also observed in the presence of cholesterol in the culture media (data not shown).

Systemic Administration of Lovastatin Does not Reduce Cholesterol Levels in the Brain

We decided to expand on the results obtained from cultured neurons by moving to an *in vivo* model. Rats were fed a diet containing high cholesterol (1%) or lovastatin (0.04%) for 5 days, and cholesterol levels were evaluated in the liver and in different regions of the brain. We expected the high-cholesterol diet to cause an increase in hepatic cholesterol (42%) (Figure 3A). However, cholesterol levels in the brain remained unchanged in the cerebellum, cerebral cortex, and hippocampus (Figure 3B). As has been previously reported, basal cholesterol levels were significantly higher in the brain than in the liver. To determine whether lovastatin consumption has an impact on brain cholesterol, rats were fed with lovastatin for 5 days. While this resulted in a significant reduction (30%) in hepatic cholesterol levels, there was not a significant change in the cholesterol concentration of any brain region studied (Figure 3A, B).

Systemic Administration of Lovastatin Increases HMGCR and SREBP-2 Levels in the Rat Brain

Because lovastatin induced a significant increase of HMGCR in differentiated neuroblastoma cells, we next investigated whether there was a similar regulation *in vivo*. Consistent with reports from Ness et al. [55], we found that rats fed 0.04% lovastatin for 5 days showed a marked increase in hepatic expression of HMGCR compared with rats fed a regular diet (Figure 4A). Although lovastatin did not change cholesterol levels in the brain, there was a significant increase in the HMGCR enzyme (Figure 4A). The administration of dietary cholesterol did not modify the levels of HMGCR in the liver or in the brain (Figure 4A). Together, these results demonstrate that *in vivo*, lovastatin exerts its regulatory effects in the brain at the level of HMGCR protein expression, without affecting cellular cholesterol levels. In addition to the effect of lovastatin on the expression of HMGCR, the mature form of SREBP-2 was also increased, particularly in the liver, the cerebral cortex and the hippocampus (Figure 4B).

Differential Effects of a HFD and Lovastatin on APP Levels and A β Production

Because statins have been proposed as alternative drugs for the treatment of AD due to the relationship between high cholesterol and A β production, we decided to investigate whether lovastatin regulates the level and metabolism of APP *in vivo* and *in vitro*. For the *in vitro* studies, differentiated human MSN cells expressing human APP₆₉₅ were exposed to lovastatin 10 μ M in the presence of serum or 2 μ M in the serum-free condition for 5 days. A significant increase was observed in APP levels in both conditions (Figure 5A). Unexpectedly, levels of secreted A β _[1–42] and A β _[1–40] were significantly reduced (11% and 27%, respectively) (Figure 5B). In contrast, rats fed with lovastatin for 5 days did not present elevated levels of APP or changes in A β _[1–40] in either cerebral region (Figure 5D). Rats fed with a HFD displayed a significant increase in APP in the cerebral cortex and hippocampus (21% and 23%, respectively) (Figure 5C) as well as a significant increase (45%) in A β _[1–40] in the cerebral cortex (Figure 5D).

Figure 2 Lovastatin induces upregulation of HMGCR and SREBP-2 activation in MSN cells. **(A)** Western blot for HMGCR and the mature form of SREBP-2 in MSN cells after Lov (2 μ M) treatment in serum-free conditions during 2 days. For HMGCR levels 100 μ g of protein from control cells and 25 μ g of protein of Lov-treated cells were loaded. The graphs show the densitometric analysis of HMGCR (two bands: ~97 and 55 kDa) and mature SREBP-2 (60 and 65 kDa) levels in MSN cells. The results are normalized with loading control (β -actin). Values are the mean $\pm$ SEM of 3 independent experiments. **(B)** Upper panel: Western blot for HMGCR in MSN cells after Lov (2 μ M) treatment in serum-free conditions during 5 days. The graph shows the densitometric analysis of HMGCR in MSN cells. The results are normalized with loading control (β -actin). Values are the mean $\pm$ SEM of 3–6 independent experiments. Lower panel: HMGCR immunofluorescence after treatment with Lov (2 μ M) in serum-free medium during 5 days. Left panel: actin stained with phalloidin (green). Right panel: HMGCR, anti-HMGCR (1:100) and Alexa Fluor-546 (1:300, red). Images were acquired at 100 $\times$. **(C)** HMGCR mRNA expression. MSN cells were incubated in serum-free medium with Lov (2 μ M) (dark column) for 18 h. Each bar represents the mean $\pm$ SEM of triplicate determinations of four experiments. * $P < 0.05$ vs. Ctrl, ** $P < 0.01$ vs. Ctrl.

Figure 3 Determination of cholesterol concentration in liver and rat brain tissues. Rats were fed a regular diet (dark columns), HFD (gray columns), and 0.04% Lov (clear columns) for 5 days. Cholesterol concentration in (A) liver and (B) brain tissues (Ctx, Cb and Hp) was determined using the Amplex Red cholesterol assay. The results are expressed as μmol of cholesterol after being normalized by protein content. The mean $\pm$ SEM of triplicate determinations of three experiments is plotted. * $P < 0.05$ vs. Ctrl.

Discussion

Although several lines of evidence support a relationship between high levels of circulating cholesterol and the risk of developing AD, the role of brain cholesterol in the expression of AD markers and the potential protective effects of statins remain unclear. We have demonstrated that a high-cholesterol diet increases the expression of APP *in vivo*. Furthermore, a high-cholesterol diet increases the expression of A β *in vivo* without affecting cholesterol levels in the brain.

In clinical situations, statins are widely used to lower plasmatic cholesterol. It has also been suggested that statins could be used for the prevention and treatment of AD due to their neuroprotective effects, including A β reduction [13,30–32], antioxidant capacities [32,33], and anti-inflammatory effects, among others. However, the implications of lowering cholesterol levels and the pleiotropic effects of statins on the CNS are not well understood. Because cholesterol and its intermediate metabolites are crucial for normal brain function, statin therapy for AD is matter of debate. In fact, there have been reports of negative consequences from treatment with statins, such as adverse cognitive effects in animals [57], psychiatric manifestations in humans [58], impairment of neurotransmission [47,59,60], altered neuroplasticity [36,38,45], and even neurotoxicity [37,42]. This study demonstrates that prolonged exposure to lovastatin, over a range of doses, induces neuronal death in MSN cells. Lovastatin-induced cell death occurred even with the addition of free cholesterol or LDL-C, suggesting that the neurotoxicity may be attributed to the pleiotropic effects associated with statins [39–42,44]. Consistent with our observations, other reports have shown that lovastatin only reduces cholesterol levels in serum-free medium. This process is accompanied by the reduction of intermediates, such as FPP and GGPP, and the reduction of protein isoprenylation in cultured neurons [61], which may contribute to the observed toxic effects of lovastatin. Although lovastatin inhibits *de novo* cholesterol synthesis, we have confirmed that cellular cholesterol levels are maintained, at least in cultured neurons. This may be the result of lipoprotein uptake from cholesterol present in the serum. An interesting finding in this work was the presence of HMGCR in neurons and its remarkable upregulation induced by lovastatin. It

is generally accepted that astrocytes are the cells that are mainly involved in cholesterol synthesis [62]. Here, we demonstrated that neurons might also participate in cholesterol regulation in the brain and that statins may modify cholesterol metabolism in these cells. *In vivo*, 5 days of lovastatin treatment was sufficient to produce a remarkable reduction in hepatic cholesterol, although brain cholesterol was not affected, similar to the results obtained from cultured cells. There are multiple reasons that could explain why lovastatin did not have an effect on total brain cholesterol levels: for example, cholesterol in the brain has greater metabolic stability and a longer half-life than cholesterol in peripheral organs [63]. It is also possible that in this model, 5 days of lovastatin treatment is not sufficient to produce levels that completely inhibit HMGCR in the brain because lovastatin concentrations in CSF (0.9–1.4 ng/mL) [50] are lower than the IC₅₀ for HMGCR inhibition in hepatocytes (2.7 ng/mL) [64]. These differences suggest that there is only partial inhibition of HMGCR in the brain in our model. We do know that lovastatin entered the brain regions analyzed due to a notable increase in the expression of the HMGCR-limiting enzyme as well as in the mature form of the transcription factor SREBP-2. The upregulation of HMGCR protein we observed is similar to that reported for the liver of rats fed with the same diet (0.04% lovastatin); in these rats, cholesterol levels were effectively reduced [55]. However, in this previous study, the authors found that reductase purified from the lovastatin-fed rats had much lower specific activity [55]. Interestingly, we observed that lovastatin exposure increased HMGCR protein levels and, paradoxically, also increased its degradation, which may indicate loss of the regulation of the enzyme by sterols or due to a mechanism unrelated to sterol concentration. In light of the fact that lovastatin inhibits synthesis of sterols and nonsterols, this result is in line with the widely held view that sterols regulate transcription and nonsterols act posttranscriptionally [65]. The notable increase in HMGCR protein and mRNA may affect the production of other biologically active intermediates in the mevalonate pathway, possibly with negative consequences for neuronal function.

High levels of circulating cholesterol have been proposed to affect the production and accumulation of A β in the brain [10–13,29]. Therefore, statins have been proposed as an alternate ther-

Figure 4 Levels of HMGCR and mature SREBP-2 in liver and rat brain tissues. **(A)** Western blots of HMGCR (~ 97 kDa) and **(B)** the mature form (~ 68 kDa) of SREBP-2 in liver and brain (Ctx, Cb and Hp). Rats received a regular diet (dark columns), a HFD (gray columns), and 0.04% Lov (clear columns) for 5 days. The graphs represent changes in HMGCR and SREBP-2 levels when normalized by a loading control (β -actin) and compared to controls. The mean $\pm$ SEM of three experiments are depicted. * $P < 0.05$ vs. Ctrl. *** $P < 0.001$ vs. Ctrl.

apy for the treatment and prevention of AD. However, the role of cholesterol in the brain and the effects of statins on APP metabolism are not known. In this study, we showed that rats fed with a HFD presented increased cholesterol in the liver but not in the brain. Additionally, we observed an important rise in the production of APP in the hippocampus and cerebral cortex. However, this was accompanied by an increase in $A\beta$ levels only in the cerebral cortex, indicating that the metabolic condition of the cortex makes

it particularly sensitive to $A\beta$ accumulation. This study is the first demonstration that only 5 days of a HFD is sufficient to change the expression and processing of APP without altering the accumulation of cholesterol in the brains of normal rats. Currently, there is no mechanistic explanation for this apparent paradox. High levels of serum cholesterol may induce vascular changes that alter BBB permeability to favor the access of reactive molecules, such as cytokines or reactive oxygen species, to the CNS [66].

Figure 5 Differential effects of lovastatin on APP and $A\beta$ levels *in vivo* and *in vitro*. Western blots of APP in MSN cells and in rat brains after Lov treatment. **(A)** MSN cells were treated with Lov 10 μ M in 10% serum or 2 μ M in serum-free media for 5 days. The results were normalized with β -actin. Graphs represent the densitometric analysis of APP levels. The mean $\pm$ SEM of three experiments is shown. **(B)** $A\beta$ levels in culture medium after Lov treatment. $A\beta_{[1-42]}$ and $A\beta_{[1-40]}$ contents are represented as fold changes with respect to control cells. The mean $\pm$ SEM of three experiments in triplicate are plotted. **(C)** Western blot of APP levels in brain tissues (Ctx, Cb and Hp). Rats were fed with a regular diet (dark columns), HFD (gray columns), and Lov 0.04% (clear columns) for 5 days. The graphs represent changes in APP levels as compared with control. The results were normalized using a loading control (β -actin) and presented as the mean $\pm$ SEM from three experiments. **(D)** $A\beta$ levels in rat brain (Ctx, Cb and Hp). The results were normalized by tissue protein. * $P < 0.05$ vs. Ctrl, ** $P < 0.01$ vs. Ctrl.

Consistent with our results, Ghribi et al. [67] reported that cholesterol-induced $A\beta$ and iron deposition was greater in the rabbit cortex than in the hippocampus and was associated with BBB disruption. Interestingly, the $A\beta$ load in the cerebral cortex is an early indicator for patients with AD [68].

Previous studies have found that statins have anti-amyloidogenic properties [13,20,28,69]. In this study, we demonstrated the differential effects of lovastatin treatment on the expression of APP and $A\beta$ *in vivo* and *in vitro*. In cultured neurons, lovastatin-mediated inhibition of HMGCR decreased $A\beta$ secretion, consistent

with the studies mentioned above. This effect was independent of the reduction in cholesterol, indicating an alternate mechanism is responsible for the effect of statins on APP metabolism. In contrast, *in vivo* lovastatin treatment produced a slight increase in APP without affecting A β production. It is possible that 5 days of lovastatin treatment was not sufficient to reach pharmacological doses that affect A β metabolism. However, the treatment was sufficient to produce a robust increase in molecules related to cholesterol metabolism, such as HMGCR and SREBP, in the cerebral cortex and in the hippocampus. Some studies have indicated that different metabolites of the mevalonate pathway (cholesterol and isoprenoids) have differential effects on APP expression and A β production. This finding would explain some of the apparent discrepancies between the *in vivo* and *in vitro* effects of lovastatin, depending on the doses reached in cells. For example, Ostrowski et al. [70] showed that the inhibition of protein isoprenylation by simvastatin and lovastatin in neuroblastoma cells causes the accumulation of APP through the inhibition of G proteins (Rho, Rac, Ras and Rab) that reduce APP C-terminal fragments and A β secretion. These results, together with our findings, suggest that the *in vivo* effect of statins on APP are extremely complex because multiple molecules altered by the inhibition of HMGCR are affected by statin treatment *in vitro* and *in vivo* [30,71,72]. Although we explored the short-term effects of lovastatin on basal A β production, other studies have shown that long-term administration of simvastatin is able to reduce levels of A β in transgenic mice that overproduce this peptide [73] and increases memory-related protein levels and synaptic plasticity [74,75]. However, AD

patients treated with simvastatin for 12 weeks did not exhibit changes in AD-related markers and exhibited only a modest decrease in brain cholesterol metabolism [76]. In conclusion, we demonstrated that lovastatin at the doses used, differentially affect the expression and production of APP and A β *in vivo* and *in vitro*. We showed that several of the effects of lovastatin-induced HMGCR inhibition have detrimental implications for neuronal survival *in vitro*. These effects are independent of a statin-induced reduction in cholesterol. *In vivo*, short-term administration of lovastatin does not directly reduce cholesterol levels; instead, it affects the mevalonate pathway, as indicated by the increase in mature SREBP-2 and upregulation of HMGCR we observed. These findings may help to elucidate some of the effects of lovastatin on cholesterol homeostasis in the brain and may provide additional evidence on the role of statins on APP expression and metabolism.

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Disclosures

The authors declare no conflict of interest.

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