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Statins and the Squalene Synthase Inhibitor Zaragozic Acid Stimulate the Non-Amyloidogenic Pathway of Amyloid-β Protein Precursor Processing by Suppression of Cholesterol Synthesis

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Abstract. Cholesterol-lowering drugs such as statins influence the proteolytic processing of the amyloid-β protein precursor (AβPP) and are reported to stimulate the activity of α-secretase, the major preventive secretase of Alzheimer's disease. Statins can increase the α-secretase activity by their cholesterol-lowering properties as well as by impairment of isoprenoids synthesis. In the present study, we elucidate the contribution of these pathways in α-secretase activation. We demonstrate that zaragozic acid, a potent inhibitor of squalene synthase which blocks cholesterol synthesis but allows synthesis of isoprenoids, also stimulates α-secretase activity. Treatment of human neuroblastoma cells with 50 μM zaragozic acid resulted in a $\sim$3 fold increase of α-secretase activity and reduced cellular cholesterol by $\sim$30%. These effects were comparable to results obtained from cells treated with a low lovastatin concentration (2 μM). Zaragozic acid-stimulated secretion of α-secretase cleaved soluble AβPP was dose dependent and saturable. Lovastatin- or zaragozic acid-stimulated increase of α-secretase activity was completely abolished by a selective ADAM10 inhibitor. By targeting the α-secretase ADAM10 to lipid raft domains via a glycosylphosphatidylinositol anchor, we demonstrate that ADAM10 is unable to cleave AβPP in a cholesterol-rich environment. Our results indicate that inhibition of cholesterol biosynthesis by a low lovastatin concentration is sufficient for α-secretase activation.

Keywords: ADAM10, α-secretase, Alzheimer's disease, lovastatin, shedding, Zaragozic acid A

INTRODUCTION

Alzheimer's disease (AD) is the most common form of neurodegenerative dementia which is characterized by extracellular amyloid plaques, intracellular neurofibrillary tangles, and neuronal dysfunction.

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Amyloid-β peptides (Aβ) derived from the amyloid-β protein precursor (AβPP) by sequential proteolytic cleavage through β- and γ- secretases are the major components of amyloid plaques. The amyloid hypothesis assumes that abnormal accumulation of Aβ in the brain is a cause of neurodegeneration and cognitive deficits in AD.

In the alternative non-amyloidogenic pathway, AβPP is cleaved within the Aβ domain by the α-secretase [1, 2]. This cleavage precludes the formation of amyloidogenic peptides and leads to the release of soluble N-terminal AβPP fragments (sAβPPα) with neuroprotective and neurotrophic properties [3–5].

Biochemical, epidemiological, and genetic aspects demonstrate a link between cholesterol levels, Aβ production, and AD. Furthermore, epidemiological data indicate that a high serum cholesterol concentration during midlife increases the risk of AD later in life [6, 7], and numerous clinical studies reported increased cholesterol levels in the blood of AD patients [8–10].

Based on these relationships between cholesterol and AD, it has been hypothesised that cholesterol-lowering drugs such as statins might have a therapeutic potential in the prevention and treatment of AD. Several early epidemiological studies suggested a protective effect of statins against the development of cognitive impairment and AD [11–13], however, other studies have not supported these findings [14–16]. These conflicting results could be related to differences in the methodological and statistical design of the studies. Nevertheless, recently published, a large cohort study indicates a reduction in the risk of AD incident by statin users [17].

In animal studies, it was shown that a cholesterol-rich diet accelerates Aβ deposition in the brain whereas the opposite effect was observed after treatment with cholesterol-lowering drugs [18–20]. *In vitro* studies demonstrated that cholesterol influences the activity of the enzymes involved in the metabolism of AβPP and in the production of Aβ. The enzymes generating Aβ, the β- and γ- secretases, operate best in a high-cholesterol environment favoring Aβ production, whereas a reduction of cellular cholesterol leads to a decrease in Aβ production [19,21–23]. Various cell culture studies showed that cholesterol reduction by statin treatment promotes the non-amyloidogenic α-secretase pathway by increasing the formation of neuroprotective sAβPPα and by decreasing Aβ production [23–25]. Despite of all these findings, the mechanism of statin action on secretases still remains unclear.

Several studies suggest that statins may regulate the α-secretase activity either by their cholesterol-lowering effects [23,24] or by impairment of the isoprenoid pathway [24–26]. Comparison of these data is complicated by the fact that studies were performed under different experimental conditions. Several factors that affect the extent of cholesterol and isoprenoid biosynthesis such as cell type, differences in statin concentration, and exposure time or overexpression of diverse AβPP isoforms, may account for some of these inconsistent results. In this regard, it is important to consider the detectable statin concentration in human serum and in the cerebrospinal fluid (CSF). The maximal concentration of statins determined in a pharmacokinetical study in CSF (0.9–1.3 ng/ml = 2.2–3.2 nM) and in serum (7.4–11.5 ng/ml = 18.3–28 nM) [27] is substantially lower than the concentrations used in most experimental conditions.

The aim of the present study was to analyze the effects of cholesterol and isoprenoid biosynthesis on α-secretase activity. Human neuroblastoma SK-N-MC cells endogenously expressing both AβPP and the α-secretase ADAM10 were treated with statins or with zaragozic acid A. This potent inhibitor of squalene synthase blocks cholesterol synthesis but allows the synthesis of isoprenoid derivatives. As depicted in Fig. 1, by using lovastatin, a squalene synthase inhibitor, and specific inhibitors of protein isoprenylation, we examined the contribution of both, the isoprenoid pathway and the cholesterol biosynthesis pathway, in α-secretase activation.

MATERIALS AND METHODS

Materials

We used the following primary antibodies: mouse IgG 6E10 detecting sAβPPα; polyclonal rabbit antibody detecting sAβPPβ (Signet, Emeryville, CA, USA); 6687 (kindly provided by C. Haass, Ludwig-Maximilians University Munich, Germany), an antibody against the C-terminus of human AβPP; anti-*myc* antibody 9E10 (hybridoma cell culture supernatant); anti-ADAM10 antibody (Chemicon International Inc., Temecula, CA, USA). Secondary anti-mouse and anti-rabbit antibodies either peroxidase-coupled or ^{35}S-labeled were from GE Healthcare Life Sciences (Freiburg, Germany). ECL detection reagent was from Pierce; methyl-β-cyclodextrin (MβCD), Zaragozic acid A, mevalonate, and lovastatin were from Sigma-Aldrich (Germany). The inhibitors applied, GM6001 (broad-spectrum metalloproteinase

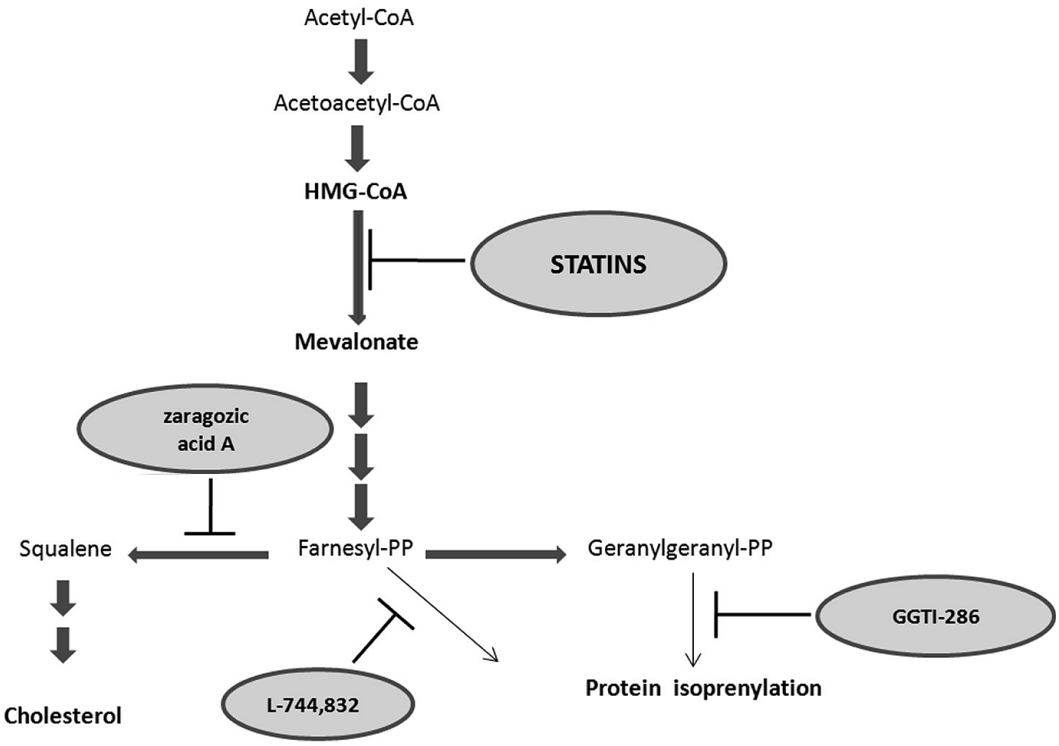

Fig. 1. Biochemical pathways leading to isoprenoid and cholesterol synthesis. Statins: inhibitors of the rate limiting enzyme of the mevalonate pathway, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Zaragozic acid A: a potent inhibitor, *in vivo* and *in vitro*, of squalene synthase which specifically inhibits cholesterol biosynthesis. Specific inhibitors of the protein isoprenylation pathways: L-744,832 – a potent and selective farnesyltransferase inhibitor; GGTI-286 – a potent and selective inhibitor of geranylgeranyltransferase.

inhibitor), GGTI-286 (geranylgeranyltransferase inhibitor), L-744,832 (farnesyltransferase inhibitor), and lactacystin (proteasome inhibitor), were from Calbiochem, and GI254023X (a preferential ADAM10 inhibitor) was from GlaxoSmithKline.

Modulation of cellular cholesterol content

SK-N-MC and HEK293 cells were grown in DMEM supplemented with 10% FCS; SH-SY5Y cells in DMEM/Ham's F-12 medium with 15% FCS. All cells were grown nearly to confluence on 10-cm dishes coated with poly-L-lysine. Lovastatin and simvastatin were converted (hydrolyzed) to their active form as described [28]. Cells for the lovastatin and zaragozic acid experiments were cultured for 24 h with DMEM supplemented with 10% lipid-deficient serum (LDS) and with indicated concentrations of lovastatin or zaragozic acid. Then the medium was replaced by serum-free DMEM containing fatty acid-free BSA (10 μg/ml) and lovastatin or zaragozic acid. Cells were incubated for 4 h and then the medium was collected and analyzed for sAβPPα or sAβPPβ as described below. For acute cholesterol depletion, HEK and HEK ADAM10-GPI cells were incubated at 37°C for 30 min with 10 mM MβCD. After washing (3x) with serum-free DMEM, cells were incubated in serum-free DMEM containing fatty acid – free BSA (10 μg/ml) for 4 h and the medium was analyzed regarding sAβPPα or sAβPPβ.

Immunoblot analysis of secreted sAβPPα and sAβPPβ

Cell supernatants were collected and proteins were precipitated with 10% (v/v) trichloroacetic acid (TCA). Immunoblot analysis of secreted sAβPPα and sAβPPβ was performed as described [29]. Briefly, for sAβPPα detection, membranes were probed with antibody 6E10 and for sAβPPβ with an anti-sAβPPβ antibody (Signet), followed by a ^{35}S-labeled or peroxidase-coupled anti-mouse or anti-rabbit antibody. The specific bands corresponding to sAβPPα or sAβPPβ were quantified by chemiluminescence or phosphoimaging using the VersaDoc system (Bio-Rad Laboratories, Mu-

nich, Germany) or the Bio-Imaging Analyzer BAS-1800 (FujiFilm Medical Systems, Duesseldorf, Germany). The protein content of each cell culture dish was determined by the Bradford method, and the values of the specific protein bands were normalized to the protein amount. The α- or β-secretase activity in treated cells was compared to the activity found in solvent treated cells.

Small interference RNA experiments

Stealth RNAi duplexes were purchased from Invitrogen, and transfections were performed according to the manufacturer's protocol. After transfection, cells were first grown for 24 h in DMEM supplemented with 10% FCS, then cultured for 24 h in DMEM supplemented with 10% LDS and 2 μM lovastatin as indicated. Cell medium was analyzed for sAβPPα and cell membranes for ADAM10 expression.

Detection of ADAM10 promoter-driven luciferase activity

The SK-N-MC cell line, stably expressing a firefly luciferase ADAM10 promoter reporter construct, was prepared as previously described [30].

Cells were grown on 60-mm diameter dishes in DMEM supplemented with 10% FCS. Cells for the lovastatin and zaragozic acid experiments were washed twice with DMEM, and then the medium was replaced with DMEM medium containing 10% LDS medium. As indicated, the medium was supplemented with 2 μM lovastatin or with 50 μM zaragozic acid A and cultured for 24 h. After this treatment, cells were lysed with lysis buffer (Promega). Firefly luciferase activities were measured using a luciferase reporter assay system (Promega) and the chemiluminescence reader Fluo Star Optima (BMG). Luciferase activity per well was normalized to the protein content (relative light units, RLU). Data are represented as mean values $\pm$ standard deviation (S.D.) from at least three independent experiments, with each experimental point performed in triplicate.

RNA preparation and real-time RT-PCR

Cells were cultured in DMEM, supplemented with 10% lipid-deficient serum (LDS) and 1 or 2 μM lovastatin, simvastatin, or cerivastatin or 50 μM zaragozic acid A as indicated. After 24 h the total RNA was isolated using the RNeasy Kit (Qiagen). Real-time RT-PCR primers used:
GAPDH_ for, 5'-GAAGGGCTCATGACCACAGTCCAT-3';
GAPDH_rev, 5'-TCATTGTCGTACCAGGAAATGAGCTT-3'; amplicon length: 437bp

ADAM10_for, 5'-CTGGCCAACCTATTTGTGGAA-3';
ADAM10_rev, 5'-GACCTTGACTTGGACTGCACTG-3'; amplicon length: 162bp

hHMG_CoA_Red_for 5'-CAAACATTGTCACCGCCATC-3'
hHMG_CoA_Red_rev 5'-TGCCATAAGTGACAATTCCCC-3'; amplicon length: 305bp

hBACE_Reti_for 5'-GTTATCATGGAGGGCTTCTACGTT-3'
hBACE_Reti_rev 5'-GCTGCCGTCCTGAACTCATC-3'; amplicon length: 101bp

hNep_Reti_for 5'-ATAGTCTTCCCAGCCGGCATT-3'
hNep_Reti_rev 5'-ATCGAAGCCATGGGTGATTTC-3'; amplicon length: 117bp

SREBP2_Reti_for 5'-CCAGAATGCAGCTACTAGCTTTCA-3'
SREBP2_Reti_rev 5'-CCATTGGCCGTTTGTGTCA-3'; amplicon length: 141bp

Real-time RT-PCR reactions were performed by using the one-step QuantiTect SYBR Green RT-PCR-Kit (Qiagen), the ABIPrism 7000 (Applied Biosystems), 100 ng RNA and sequence specific primer pairs (0.5 μM of each primer). The mRNA quantity was calculated by the $\Delta\Delta$Ct method. Compared to solvent treated cells, the mRNA amount of the housekeeping gene GAPDH remained unchanged in treated cells and was used for normalization.

Generation of HEK ADAM10-GPI expressing cells

The coding region of the ADAM10 transmembrane and cytoplasmic domains was replaced by a GPI coding sequence by insertion of a synthetic oligonucleotide adapter encoding the amino acid sequence PDHSAATKPSLFLFLVSLLHIFFK. For stable expression in HEK 293 cells, the vector pIRES-neo2 (Clontech) containing the mycADAM10-GPI nucleotide sequence was used. Transfection of cells was performed using Lipofectamin2000 and HEK ADAM10-GPI cells were selected with G418.

Flotation gradients

HEK ADAM10-GPI cells were grown to confluence, and then were either depleted of cholesterol by incubation with 10 mM MβCD for 30 min or treated with medium, then washed with ice-cold PBS, scraped in PBS, and spun down at 750 x g at 4°C. Fractionations were carried out as described [31]. Twelve fractions from the top of the gradient were collected and proteins were precipitated with TCA. Myc-tagged ADAM10-GPI was detected by immunoblot with anti-myc antibody 9E10 and caveolin with an anti-caveolin-1 antibody (Santa Cruz Biotechnology) followed by peroxidase-coupled secondary antibodies and a chemiluminescent detection system.

Cell membrane preparation

The cells were suspended in buffer (20 mM HEPES pH 7.4) at 4°C, then sonicated for 20 s and centrifuged at 100,000 x g at 4°C for 1 h. The pellet was resuspended in the same buffer, sonicated and centrifuged under the same condition. The membrane pellet was resuspended in 20 mM HEPES, pH 7.4 and stored at −80°C. Protein content was determined by the Bradford method.

Immunoblot analysis of ADAM10 and AβPP

Aliquots containing 200 μg (ADAM10) or 20 μg (AβPP) of cell membrane proteins were separated by SDS/PAGE on 10% gels (ADAM10) or Nu-PAGE gels (AβPP) and blotted onto PVDF membranes. Membranes were probed with specific antibodies against AβPP (6687) or ADAM10 (Chemicon) followed by a ^{35}S-labeled (ADAM10) or peroxidase-coupled (AβPP) anti-rabbit antibody. The specific bands were quantified by phosphoimaging (ADAM10) or chemiluminescence (AβPP) using the Bio-Imaging Analyzer BAS-1800 or the VersaDoc-Imaging system.

Cholesterol determination

Cells were lysed in reaction buffer (5 mM cholic acid, 0.1% Triton X-100 in PBS, pH 7.4) at 4°C for 30 min, then centrifuged at 16 000 x g at 4 °C for 30 min. The cellular cholesterol content was measured using the Amplex Red cholesterol kit (Invitrogen) according to the manufacturer's protocol, except that cholesterol esterase was omitted from the reaction mixture. Fluorescence was measured with the micro plate reader Fluo Star Optima (BMG). The cholesterol levels are expressed as μg cholesterol/mg protein. Protein content was determined by the Bradford method.

Statistical analysis

The results are expressed as percentage relative to control (unstimulated cells) and are the averages ± SD of at least three independent experiments performed in duplicate. Statistical significance between control cells and treated cells was determined by using one-way ANOVA/Bonferroni post hoc test analysis or unpaired Student's t test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

Statins as activators of the non-amyloidogenic pathway of AβPP processing

Various statin concentrations ranging from 1 to 20 μM have been described in literature for the deprivation of cellular cholesterol. It is well known that statins can reduce cell proliferation by inhibition of protein isoprenylation. Therefore, we first determined the influence of the lovastatin concentration on cell viability and proliferation by using the MTT assay (data not shown). We found that a lovastatin concentration higher than 5 μM decreases SK-N-MC cell viability; therefore 1 or 2 μM lovastatin was applied in all further experiments.

For the deprivation of cellular cholesterol by lovastatin, we used conditions previously described [23]. Cells were cultured in medium containing 10% lipid-deficient serum for 24 h; this treatment resulted in ∼25–35% reduction of cholesterol content compared to cells cultured in regular medium. Addition of 1–2 μM lovastatin to the lipid-deficient medium further reduced the amount of cellular cholesterol by ∼30%.

Treatment of SK-N-MC and SH-SY5Y cells with 1 or 2 μM lovastatin resulted in activation of the α-secretase: sAβPPα secretion from SK-N-MC cells was increased more than 2 fold (Fig. 2A) and ∼1.8 fold from SH-SY5Y (Fig. 2B) compared to untreated cells. At the same time, no influence on sAβPPβ secretion was detected (Fig. 2A,B).

Then we examined whether this lovastatin-induced increased production of sAβPPα was a result of α-secretase metalloproteinase activation. SK-N-MC cells were incubated either with the broad-spectrum metalloproteinase inhibitor GM6001 (50 μM) or the ADAM10 preferential inhibitor GI254023X (30 μM). We observed a strong inhibition of the lovastatin induced sAβPPα secretion with both inhibitors (Fig. 3A).

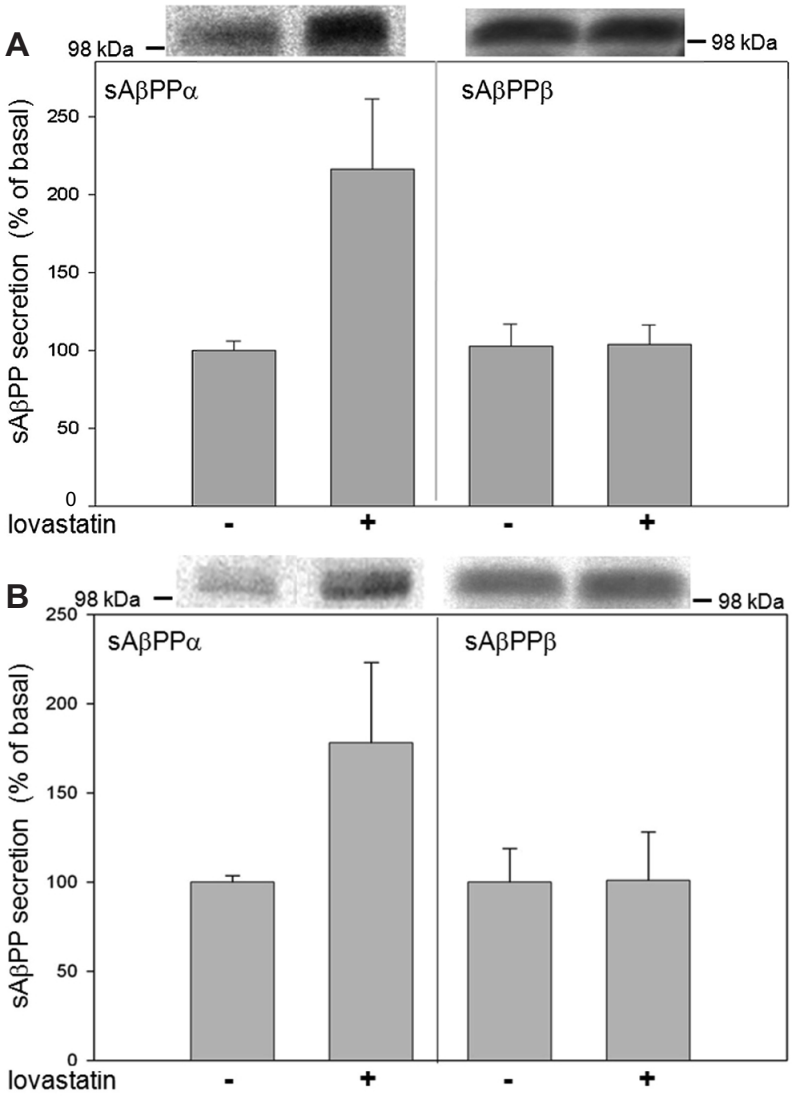

Fig. 2. Effect of lovastatin on AβPP processing. Influence of lovastatin on the secretion of sAβPPα and sAβPPβ from human SK-N-MC (A) and human SH-SY5Ycells (B). In all experiments, cells were cultured in lipid-deficient medium for 24 h in the presence of 1 μM (SH-SY5Y cells) or 2 μM lovastatin (SK-N-MC cells). Then the medium was replaced with serum-free DMEM containing fatty acid-free BSA (10 μg/ml) and lovastatin. The cells were incubated for 4 h, then the medium was collected, proteins were precipitated and subjected to immunoblot analysis with respective primary antibodies followed by ^{35}S-labeled secondary antibodies as described in Materials and Methods. The specific bands corresponding to sAβPPα or sAβPPβ were quantified with the Bio-Imaging analyzer model BAS-1800 and normalized to the cellular protein content. Blots are representative for at least three independently performed experiments per treatment. Quantifications show the mean $\pm$ SD; values from solvent-treated cells were set to 100%.

Therefore, our results clearly show that the elevated production of sAβPPα after lovastatin treatment was due to the increased α-secretase activity and was not caused by a vesicular release of preformed sAβPPα. These results suggest an involvement of ADAM10 in the lovastatin-induced α-secretase cleavage of AβPP but do not exclude the participation of other proteinases from this process.

To confirm that ADAM10 participates in lovastatin-induced α-secretase activation, RNAi-mediated knockdown of ADAM10 was performed. Lovastatin- induced sAβPPα secretion (Fig. 3C, lane 3) was completely reduced (Fig. 3C, lane 4) after efficient ADAM10 depletion (Fig. 3D). Previously, we have shown that lovastatin treatment increased the protein level of stably overexpressed bovine ADAM10 in HEK cells [23];

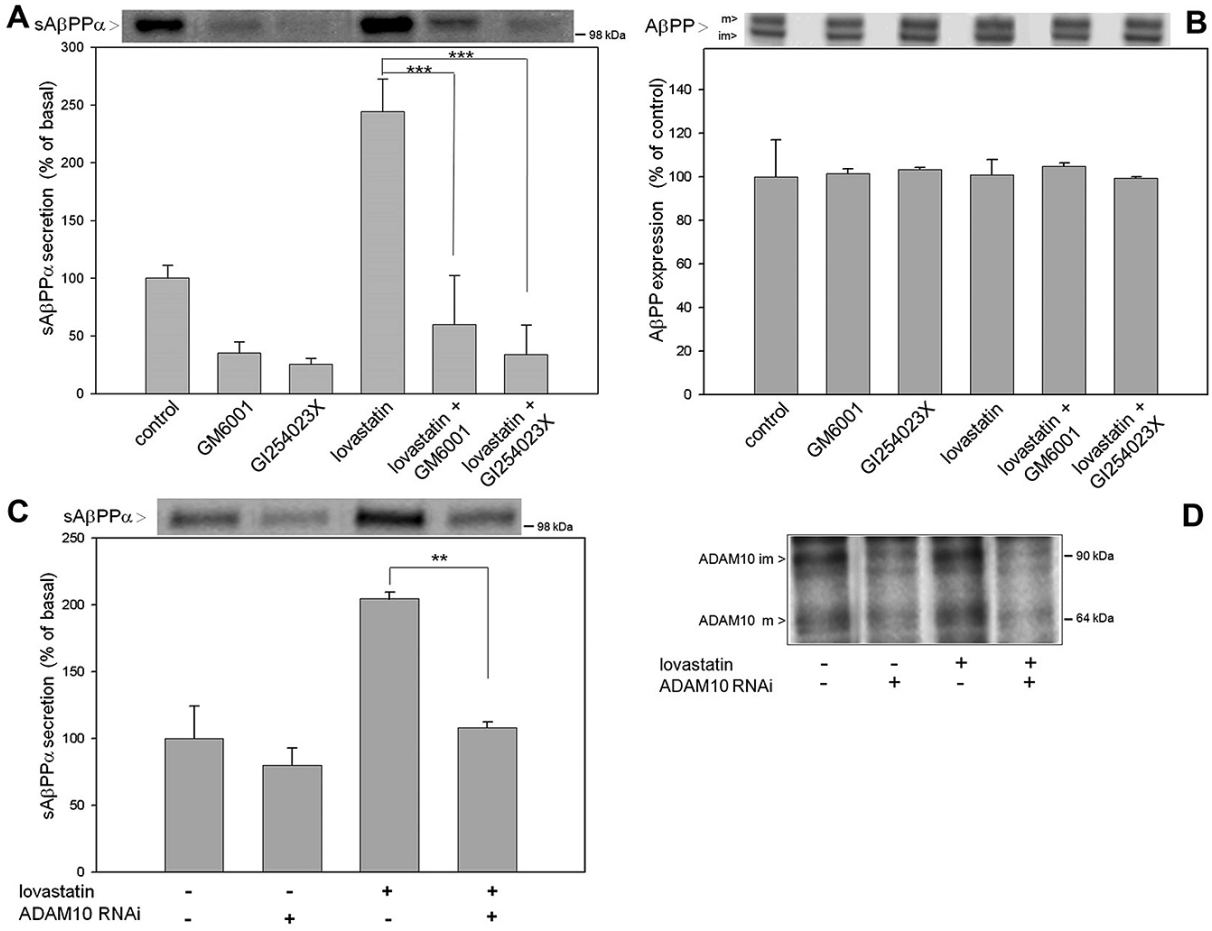

Fig. 3. Effect of hydroxamate metalloproteinase inhibitors and of RNAi-mediated knockdowns on lovastatin-induced α-secretase activity. A) Effect of metalloproteinase inhibition on sAβPPα secretion. SK-N-MC cells cultured in lipid-deficient medium for 24 h in the presence of 2 μM lovastatin were first pre-incubated for 30 min with inhibitor (50 μM GM6001 or 30 μM GI254023X as indicated), then the medium was replaced by serum-free medium containing 2 μM lovastatin and inhibitor. After 4 h, the medium was collected, and secreted sAβPPα was detected and quantified as described in Fig. 2, except that specific bands corresponding to sAβPPα were quantified by chemiluminescence. B) Analysis of AβPP expression in cells described in A. Mature [m] and immature [im] forms of AβPP are indicated by arrows. Detection and quantification of AβPP was performed as described in Materials and Methods. C) Effects of RNAi-mediated ADAM10 knockdown on the lovastatin-induced sAβPPα secretion. SK-N-MC cells were transfected with stealth RNAi oligonucleotide duplexes (Invitrogen) targeting ADAM10, as a control, cells were transfected with a stealth RNAi control duplex. Experiments were performed 48 h after transfection, cell medium was analyzed for sAβPPα. D) Effect of RNAi-mediated knockdowns on ADAM10 expression. Cell membranes (30 μg of protein per lane) were analyzed for ADAM10 expression with a suitable antibody as described in Materials and Methods. Values are means $\pm$ SD; one-way ANOVA/ Bonferroni post hoc test.

now we examined whether lovastatin treatment also influences endogenously expressed ADAM10 in SK-N-MC cells. We observed an increase of $\sim$20% of the mature form and of $\sim$30% of the proform of endogenous ADAM10 after treatment with 2 μM lovastatin (Fig. 4A and 4B). A slightly increased amount of endogenous ADAM10 could also be detected in SH-SY5Y cells after treatment with lovastatin (Fig. 4A). Treatment of SK-N-MC cells with 2 μM lovastatin or with inhibitors did not affect levels of the AβPP holoprotein (Figs 3B and 4C).

To examine whether increased α-secretase activity was caused by altered ADAM10 gene expression, mRNA levels of ADAM10 were determined in SK-N-MC and SH-SY5Y cells after lovastatin treatment. We did not observe any influence on the transcription level of the ADAM10 mRNA whereas the amount of HMGCoA reductase mRNA was significantly increased under the same experimental conditions (Fig. 4D, E). The same results were obtained after treatment of SH-SY5Y cells

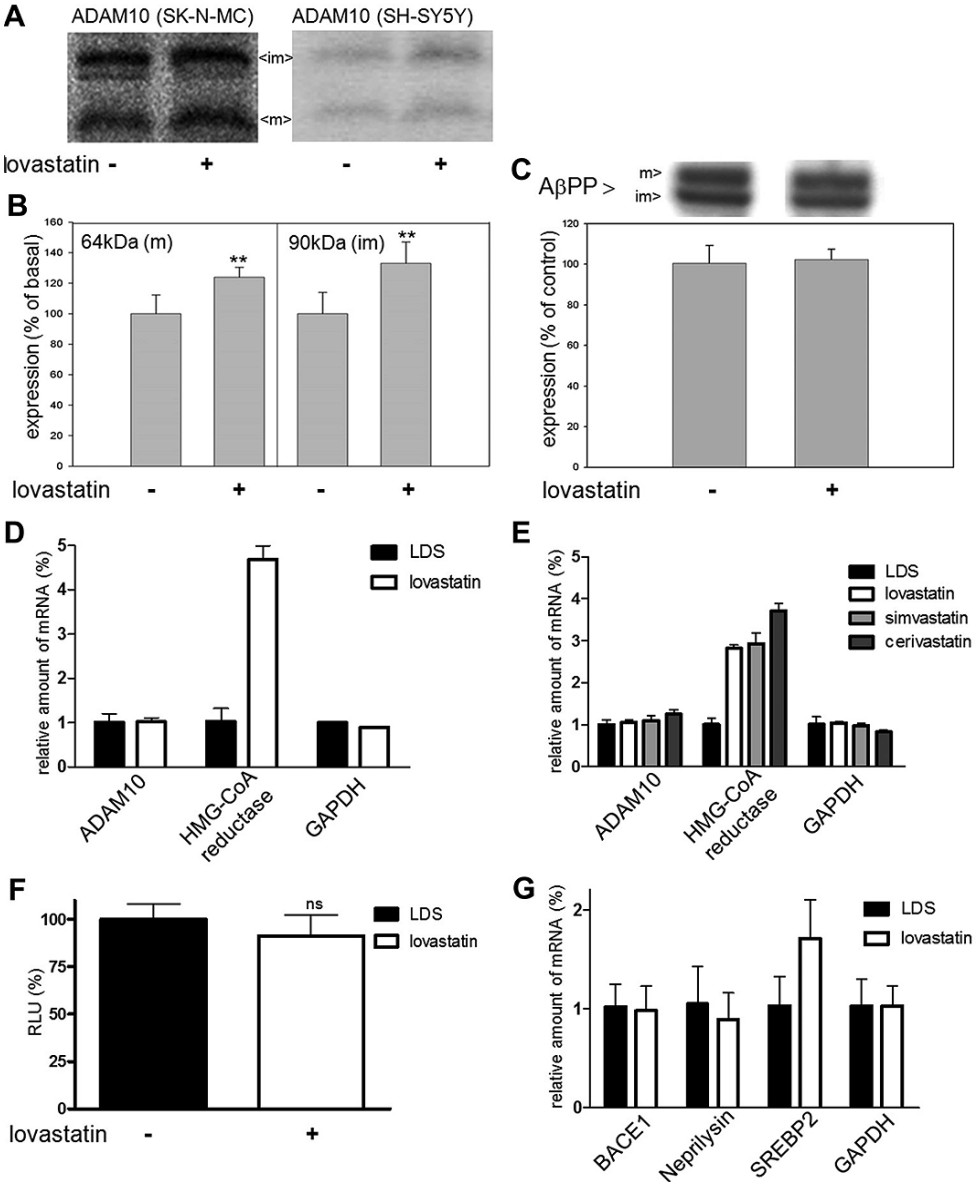

Fig. 4. Expression of the α-secretase ADAM10 and the AβPP holo-protein after treatment of cells with statins. A) Western blot analysis for detection of endogenous ADAM10 in lovastatin treated SK-N-MC and SH-SY5Y cells. B) Quantification of ADAM10 expression in lovastatin and mock treated SK-N-MC cells. C) Expression and quantification of AβPP expression in treated SK-N-MC cells. Cellular membrane proteins (200 μg for ADAM10 detection or 20 μg for AβPP detection) of each sample prepared from SK-N-MC or SH-SY5Y cells which have been cultured for 24 h in lipid-deficient medium in the absence or presence of lovastatin were analyzed by immunoblotting. Mature [m] and immature [im] forms of ADAM10 (A) and AβPP (C) are indicated by arrows. Detection and quantification of ADAM10 (A, B) and AβPP (C) was performed as described in Materials and Methods. The radioactive bands corresponding to ADAM10 were quantified with the Bio-Imaging analyzer model BAS-1800. Values are means $\pm$ SD; unpaired Student's t test ($^{**}P < 0.01$). D,E) Quantification of ADAM10 mRNA levels by quantitative Real-time RT-PCR. SK-N-MC (D) and SH-SY5Y (E) cells were cultured in lipid-deficient medium for 24 h in the absence or presence of 2 μM lovastatin (D) or 1 μM lovastatin, simvastatin or cerivastatin (E). Quantification of ADAM10 mRNA levels was performed as described in Materials and Methods. GAPDH mRNA was used for normalization and HMG-CoA-reductase mRNA as positive control. F) Effect of lovastatin on the ADAM10 promoter activity. SK-N-MC cells stably expressing a hybrid ADAM10 promoter/firefly luciferase reporter gene were cultured in lipid-deficient medium for 24 h in the absence or presence of 2 μM lovastatin. Quantification of firefly luciferase activity was performed as described under Materials and Methods and was normalized to the cellular protein content. G) Quantification of endogenous BACE1 and neprilysin mRNA levels by Real-time RT-PCR, SREBP2 mRNA was quantified as a positive control, and GAPDH mRNA was used for normalization.

with simvastatin and cerivastatin (Fig. 4E).

We also studied whether the activity of the human ADAM10 gene promoter can be influenced by treatment with lovastatin. For this purpose, stably transfected SK-N-MC cells expressing a hybrid ADAM10 promoter/firefly luciferase reporter gene were incubated in the absence or presence of 2 μM lovastatin in lipid deficient medium. Cells were lysed after 24 h and tested for luciferase activity and cholesterol levels. Lovastatin treatment reduced cholesterol by $\sim$36% but had no effect on the ADAM10 promoter activity (Fig. 4F).

Several in *vitro* and *in vivo* studies demonstrated that lovastatin reduces the generation of Aβ peptides. Therefore we examined whether lovastatin affects the gene expression of the β-secretase BACE1 and the Aβ degrading enzyme neprilysin. The mRNA levels of both enzymes were not regulated but the mRNA level of SREBP2 (sterol regulatory element-binding protein-2), used as a positive control, was increased (Fig. 4G).

Investigation whether the lactone form of lovastatin contributes to α-secretase activation

Statins exist either in an acid or lactone form. Only the acidic form acts as HMG-CoA-reductase inhibitor that blocks cholesterol biosynthesis, whereas the lacton form of lovastatin might be involved in the inhibition of proteasome-mediated protein degradation. It has been hypothesized that statins affect the proteasome activity because both the statin lacton form and the proteasome inhibitor lactacystin contain a β-lactone ring, which is essential for lactacystin-mediated proteasome inhibition [32]. Membrane proteins are frequently degraded in lysosomes but alternatively can also be degraded by proteasomal proteolysis [33]. Therefore we analyzed whether impaired proteasome-mediated degradation of the α-secretase ADAM10 could be responsible for the increased amount of this protein after lovastatin treatment.

We first examined whether the β-lactone ring of lovastatin affects α-secretase activity. For this purpose, cells were treated for 24 h with hydrolyzed (mostly acid form) and non-hydrolyzed (mostly β-lactone ring) lovastatin. To control whether the ratio between acidic and lactone lovastatin was altered under our experimental conditions, the cellular cholesterol levels were determined. Treatment of cells with hydrolyzed lovastatin resulted in a two-fold increase in sAβPPα secretion and in $\sim$30% reduction of cholesterol. By treatment with non-hydrolyzed lovastatin, we observed a weaker enhancement ($\sim$50%) of sAβPPα secretion (Fig. 5A) and only a slight reduction (16%) of cellular cholesterol (Fig. 5B). The expression of full-length AβPP was not influenced after these treatments (Fig. 5C). All statins interconvert *in vivo* and also in cell culture conditions and equilibrium between the acid and lactone forms is reached. Therefore it is difficult to determine the ratio between both forms exactly. Since activation of α-secretase inversely correlates with the cholesterol level, it seems that only the acid form of lovastatin, which is responsible for the cholesterol lowering properties of lovastatin, increases the α-secretase activity and that the β-lactone ring does not contribute to this effect.

To examine whether proteasome-mediated degradation influences α-secretase stability, we also studied the effect of the proteasome inhibitor lactacystin on sAβPPα secretion from SK-N-MC cells. Our results clearly showed that inhibition of proteasomal activity did not affect the sAβPPα secretion and therefore probably had no influence on the stability of the α-secretase ADAM10 (Fig. 5D).

α-Secretase activation under low lovastatin concentration is independent from the isoprenoid pathway

Statins can inhibit biosynthesis of cholesterol and isoprenoids. Several reports described the involvement of the isoprenoid pathway in AβPP proteolytic processing after statin treatment [24,25]. To investigate the involvement of the isoprenoid pathway in statin-mediated α-secretase activation, SK-N-MC cells were treated with lovastatin in the presence of 200 μM mevalonate or with farnesyltransferase (L-744,832) and geranylgeranyltransferase (GGTI-286) inhibitors. This concentration of mevalonate only allows restoration of the isoprenoid pathway, without influencing the blockade of cholesterol biosynthesis [34]. The increased α-secretase activity caused by lovastatin was not abolished by simultaneous treatment of cells with 2 μM lovastatin and 200 μM mevalonate (Fig. 6A, lane 2 and 3). This result showed that the lovastatin-induced activation of α-secretase is maintained under conditions where the isoprenoid pathway is unaffected.

To confirm that treatment with 200 μM mevalonate selectively reconstitutes only the isoprenoid pathway, we investigated RhoA-GTPase isoprenylation. A statin-mediated inhibition of RhoA isoprenylation has been shown to cause the loss of its normal membrane association [35,36].

To investigate RhoA localization, SK-N-MC cells were cultured in lipid-deficient medium for 24 h under

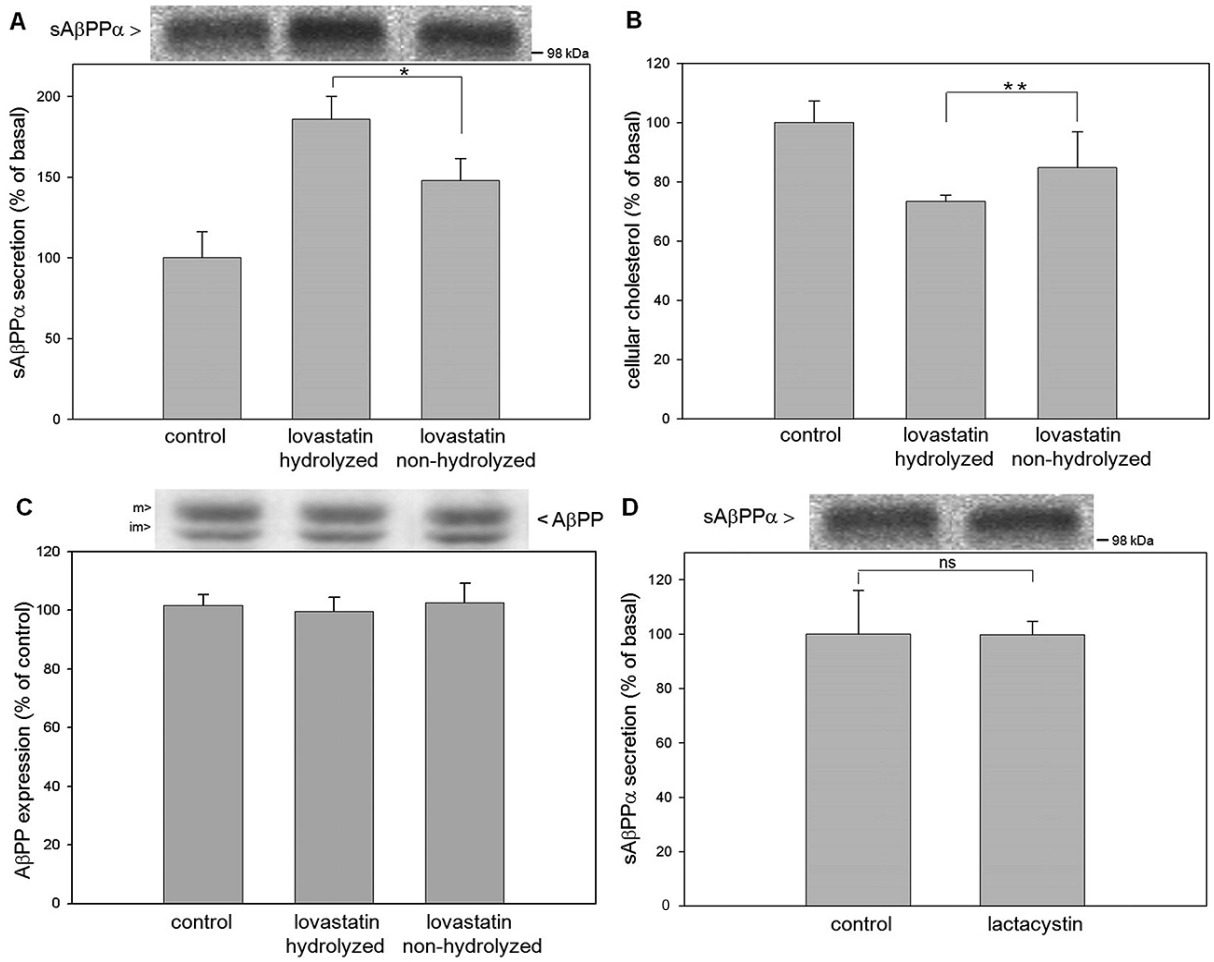

Fig. 5. Effect of acidic and lactone lovastatin forms on the α-secretase activity. A) SK-N-MC cells were incubated for 24 h in lipid deficient medium in the absence (lane 1) or in the presence of 2 μM hydrolyzed lovastatin (lane 2) or 2 μM non-hydrolyzed lovastatin (lane 3), then the medium was analyzed for secreted sAβPPα as described in Materials and Methods. B) Quantification of cellular cholesterol of the cells described in figure part A. C) Quantification of AβPP expression after treatment of cells with hydrolyzed and non-hydrolyzed lovastatin. D) Effect of lactacystin on the α-secretase activity. SK-N-MC cells were cultured for 24 h in lipid deficient medium in the absence (lane 1) or in the presence (lane 2) of 2 μM lactacystin. The medium was analyzed for secreted sAβPPα as described above. Values are means $\pm$ SD; one-way ANOVA/Bonferroni post hoc test (A, B) or unpaired Student's t test (D).

various conditions: (i) in the absence or in the presence of 2, 10, and 20 μM lovastatin and (ii) in the presence of 2, 10, and 20 μM lovastatin and 200 μM mevalonate. Subsequently, membrane fractions were analyzed for RhoA association in the membrane and cells were also analyzed for their cholesterol content.

Treatment of cells with 2 μM lovastatin or simultaneous treatment with 2 μM lovastatin and 200 μM mevalonate did not influence the membrane association of RhoA (Fig. 6B, lane 2 and 3), indicating that RhoA-isoprenylation is not affected by 2 μM lovastatin. Treatment with 10 and 20 μM lovastatin resulted in the reduction of RhoA membrane-association which is caused by inhibition of isoprenylation (Fig. 6B, lane 4 and 6). This effect was reversed by addition of 200 μM mevalonate (Fig. 6B, lane 5 and 7).

The cellular cholesterol content was reduced by $\sim$35 % by all applied lovastatin concentrations and supplementation with 200 μM mevalonate was insufficient to restore cholesterol biosynthesis (Fig. 6C). This experiment clearly shows that 2 μM lovastatin only inhibits cholesterol biosynthesis and that 200 μM mevalonate only reconstitutes the isoprenoid pathway.

To test whether protein isoprenylation may also be involved in α-secretase activation under certain conditions, cells were treated with farnesyltransferase (L-

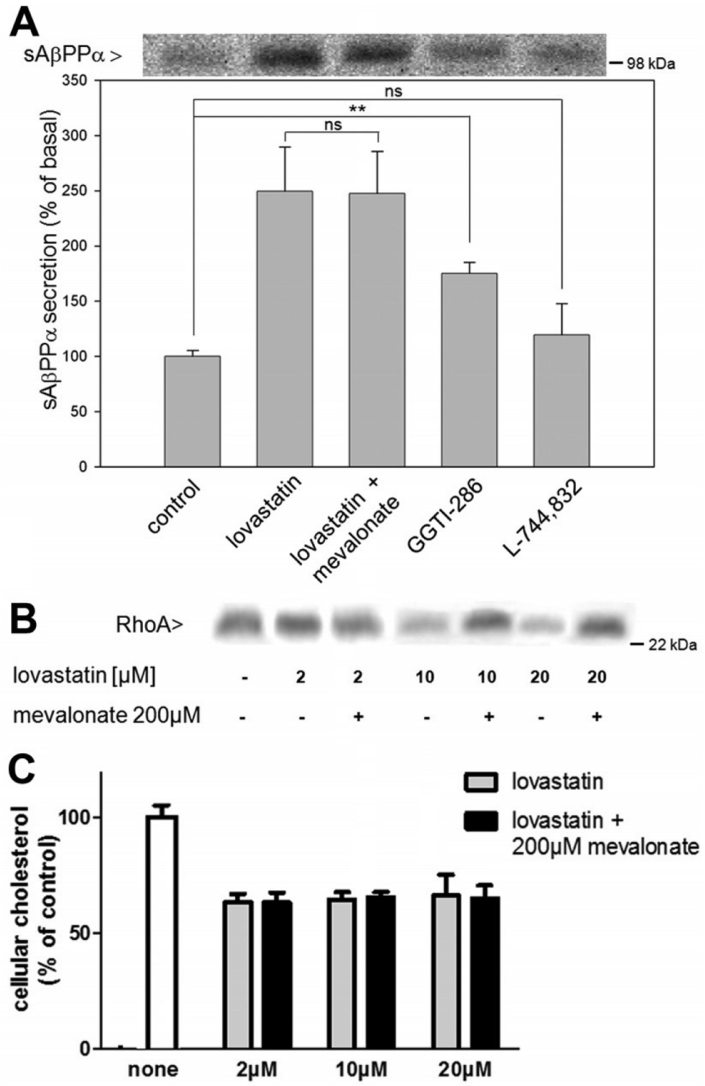

Fig. 6. Investigation of the involvement of the isoprenoid pathway to α-secretase activation. A) SK-N-MC cells were cultured for 24 h in lipid-deficient medium in the presence of 2 μM lovastatin (lane 2); 2 μM lovastatin and 200 μM mevalonate (lane 3); 10 μM geranylgeranyltransferase inhibitor (GGTI-286) (lane 4); 10 μM farnesyltransferase inhibitor (L-744,832) (lane 5) or only in lipid-deficient medium (lane 1). The medium was analyzed for secreted sAβPPα as described in Fig. 2. Values are means $\pm$SD; one-way ANOVA/Bonferroni post hoc test. B) RhoA abundance in membrane fractions of cells treated with lovastatin or with lovastatin and mevalonate. SK-N-MC cells were cultured in lipid-deficient medium for 24 h in the absence or in the presence of 2, 10, and 20 μM lovastatin or of lovastatin and 200 μM mevalonate as indicated. Then 50 μg of the membrane protein fractions were analyzed by immunoblotting. Detection of RhoA was performed with antibody 26C4 (Santa Cruz). C) Quantification of cellular cholesterol of cells described in figure part B.

744,832, 10μM) and geranylgeranyltransferase (GGTI-286, 10 μM) inhibitors. About a 1.6 fold increased sAβPPα secretion was observed after treatment of SK-N-MC cells with the geranylgeranyltransferase inhibitor (Fig. 6A, lane 4) while treatment with the farnesyltransferase inhibitor had no effect (Fig. 6A, lane 5). Thus, prevention of protein prenylation by geranylgeranyltransferase seems to contribute to α-secretase activation.

Effect of the squalene synthase inhibitor zaragozic acid A on the α-secretase activity

To confirm our results that lovastatin-induced α-secretase activation is mediated by inhibition of the sterol pathway, cells were treated with a squalene synthase inhibitor. Squalene synthase (SQS) is the enzyme that determines the switch towards sterol biosynthesis and acts downstream of mevalonate (Fig. 1).

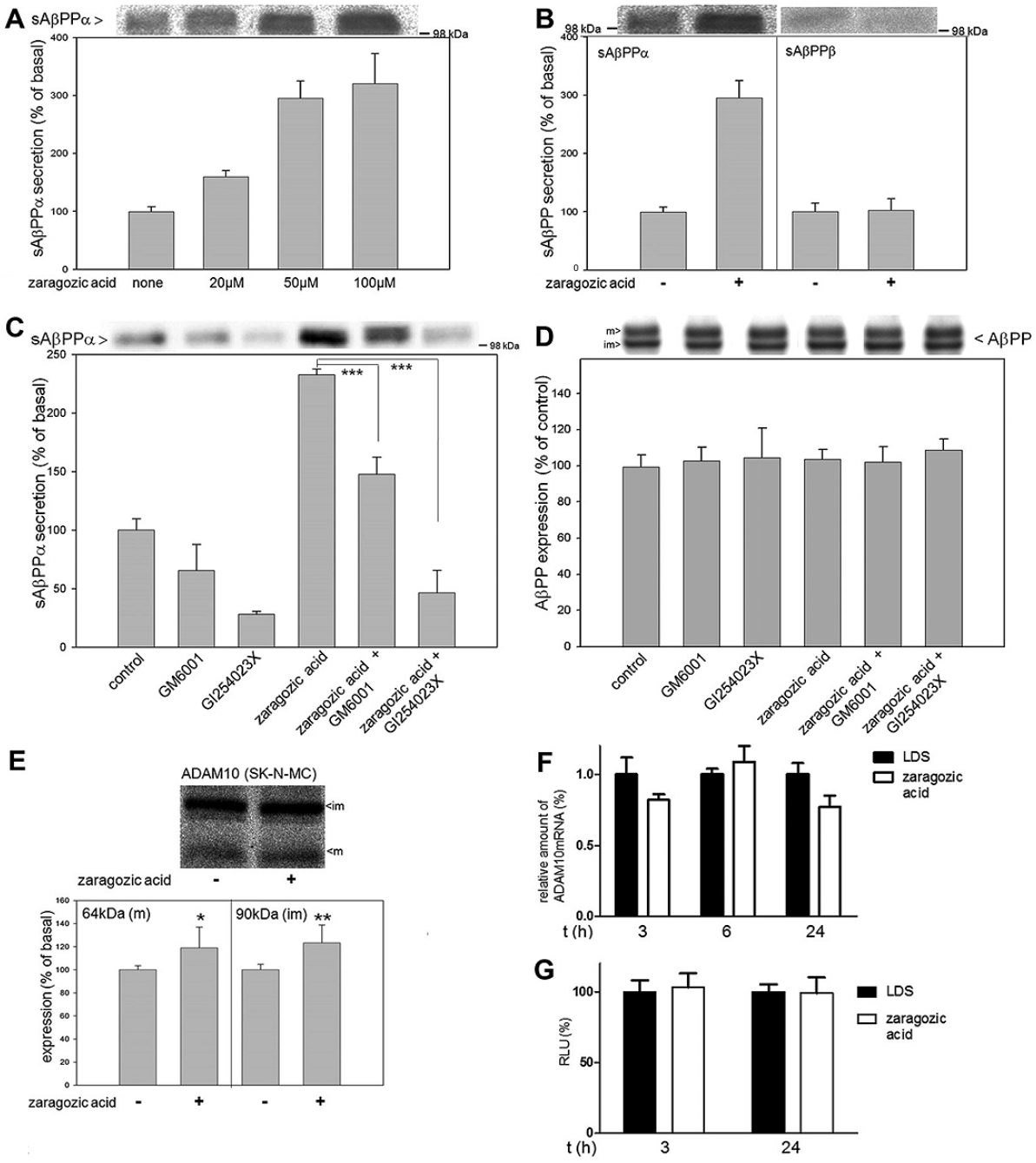

Fig. 7. Influence of the squalene synthase inhibitor zaragozic acid A on AβPP processing. A) Zaragozic acid-induced dose-dependent sAβPPα secretion. SK-N-MC cells were cultured for 24 h in lipid-deficient medium in the absence or in the presence of increasing ZA concentrations (20, 50, 100 μM), then the medium was analyzed for secreted sAβPPα as described in Fig. 2. B) Effect of ZA on sAβPPα and sAβPPβ secretion. Cells were treated for 24 h with 50 μM zaragozic acid, then the medium was analyzed for secreted sAβPPα and sAβPPβ as described above. C) Effect of metalloproteinase inhibitors on zaragozic acid-induced α-secretase activity. Experiments were performed as described in Fig. 3A only lovastatin was replaced by 50 μM ZA. D,E) Expression and quantification of the AβPP holo-protein (D) and the α-secretase ADAM10 (E) after treatment of SK-N-MC cells with 50 μM zaragozic acid. Detection and quantification of ADAM10 and AβPP was performed as described in Fig. 4 and in Materials and Methods. F) Quantification of ADAM10 mRNA levels was performed as described in Materials and Methods. G) Effect of zaragozic acid on the ADAM10 promoter activity. Experiments were performed as described in Fig. 4F only lovastatin was replaced by 50 μM ZA. Values are means $\pm$ SD; one-way ANOVA/ Bonferroni post hoc test or unpaired Student's t test.

Zaragozic acid A (ZA) is a potent inhibitor of squalene synthase, which significantly reduces cholesterol biosynthesis in hepatic cells and in the mouse [37]. We found that zaragozic acid-stimulated secretion of sAβPPα from SK-N-MC cells was dose-dependent and saturable with an EC50 value of 27 μM (Fig. 7A). Results from MTT assays showed that 10–100 μM ZA had no influence on cell viability (data not shown). Treatment of SK-N-MC cells with 50 μM ZA in lipid deficient medium reduced the cholesterol level by $\sim$30% and enhanced the sAβPPα secretion $\sim$3 fold but had no influence on the secretion of sAβPPβ (Fig. 7B). This zaragozic acid-induced α-secretase activation was strongly reduced by the metalloproteinase inhibitors GM6001 (50 μM) and GI254023X (30 μM). The strong inhibition ($\sim$80%) observed for the ADAM10 selective inhibitor GI254023X suggests an involvement of ADAM10 in zaragozic acid-induced α-secretase activation (Fig. 7C). Zaragozic acid and the applied inhibitors had no influence on AβPP expression (Fig. 7D).

However, we observed increased protein levels of both forms of the α-secretase ADAM10 ($\sim$20%) after zaragozic acid treatment (Fig. 7E). Analysis of gene expression by quantitative Real-time RT-PCR showed that the enhanced level of the ADAM10 protein was not caused by increased ADAM10 gene expression (Fig. 7F) and ZA treatment also had no effect on the ADAM10 promoter activity (Fig. 7G).

Effect of GPI-anchored ADAM10 on AβPP processing

Previously we have shown that the active form of the α-secretase ADAM10 is not localized in lipid rafts, suggesting that the α-secretase cleavage of AβPP occurs in a different membrane compartment [23]. To investigate the significance of the cholesterol environment in the cellular membrane on the α-secretase activity, we used the approach of targeting the α-secretase ADAM10 to lipid raft domains by replacing the transmembrane and C-terminal domains of ADAM10 by a glycosylphosphatidylinositol (GPI) anchor. We generated and analyzed HEK 293 cells expressing ADAM10-GPI (HEK ADAM10-GPI). The localization of ADAM10-GPI in cholesterol-rich plasma membrane microdomains was verified by isolation of lipid rafts. Both forms of ADAM10-GPI, the full-length (83 kDa) and the form lacking the prodomain ($\sim$57 kDa) were present in the fraction of low density, in the same fraction where the raft marker caveolin-1 was mostly localized (Fig. 8A, upper part). Treatment with methyl-β-cyclodextrin (MβCD) caused rafts destruction, and both ADAM10-GPI and caveolin were completely removed from these fractions (Fig. 8A, lower part). Recently another group also demonstrated that ADAM10-GPI is exclusively localized in lipid rafts [38].

To examine how targeting of ADAM10 to lipid rafts influences its α-secretase activity, we compared the secretion of sAβPPα from HEK ADAM10-GPI cells with a control cell line transfected with the empty expression vector (HEK mock) and endogenously expressing ADAM10. We observed the same amount of sAβPPα secreted from both cell lines (Fig. 8B). This suggests that targeting of ADAM10 to lipid rafts resulted in the total reduction of the α-secretase activity of this enzyme. To confirm that the localization of ADAM10-GPI in lipid rafts is responsible for the loss of α-secretase activity, cells were treated with MβCD for cholesterol depletion that causes a cholesterol reduction by $\sim$70% and a destruction of raft structures. Treatment of cells with 10 mM MβCD resulted in an increase in sAβPPα secretion: $\sim$2.7 fold in HEK mock and more than 4.1 fold in HEK ADAM10-GPI cells, respectively (Fig. 8C). Rafts destruction by cholesterol depletion resulted in an activation of the ADAM10 enzyme containing the GPI anchor: $\sim$140% more of sAβPPα was secreted from HEK ADAM10-GPI cells (Fig. 8C, lane 4) compared to HEK mock cells (Fig. 8C, lane 2).

To further confirm our observation that the cellular cholesterol concentration influences the enzymatic activity of ADAM10-GPI, HEK ADAM10-GPI, and HEK mock cells were cultured in lipid-deficient serum in the presence and absence of lovastatin. Cultivation of cells in lipid-deficient serum, which reduces cellular cholesterol $\sim$30%, resulted in ADAM10-GPI activation. Secretion of sAβPPα was increased $\sim$2.8 fold (Fig. 8D, lane 3) compared to HEK mock cells (Fig. 8D, lane 1). The enzymatic activity of ADAM10-GPI was enhanced after treatment in lipid-deficient serum, probably by rafts destruction under this condition. Treatment with lovastatin which further reduces the cholesterol content by 20–30% only affected the activity of endogenous ADAM10 but not of ADAM10-GPI. The increase of sAβPPα secretion after lovastatin treatment was in the same range in HEK ADAM10-GPI and HEK mock cells (Fig. 8D, lane 4 and 2) and therefore was caused by the activation of endogenous ADAM10. This lack of a lovastatin effect on ADAM10-GPI suggests that the C-terminus of the α-secretase ADAM10 is substantial for α-secretase activation by lovastatin.

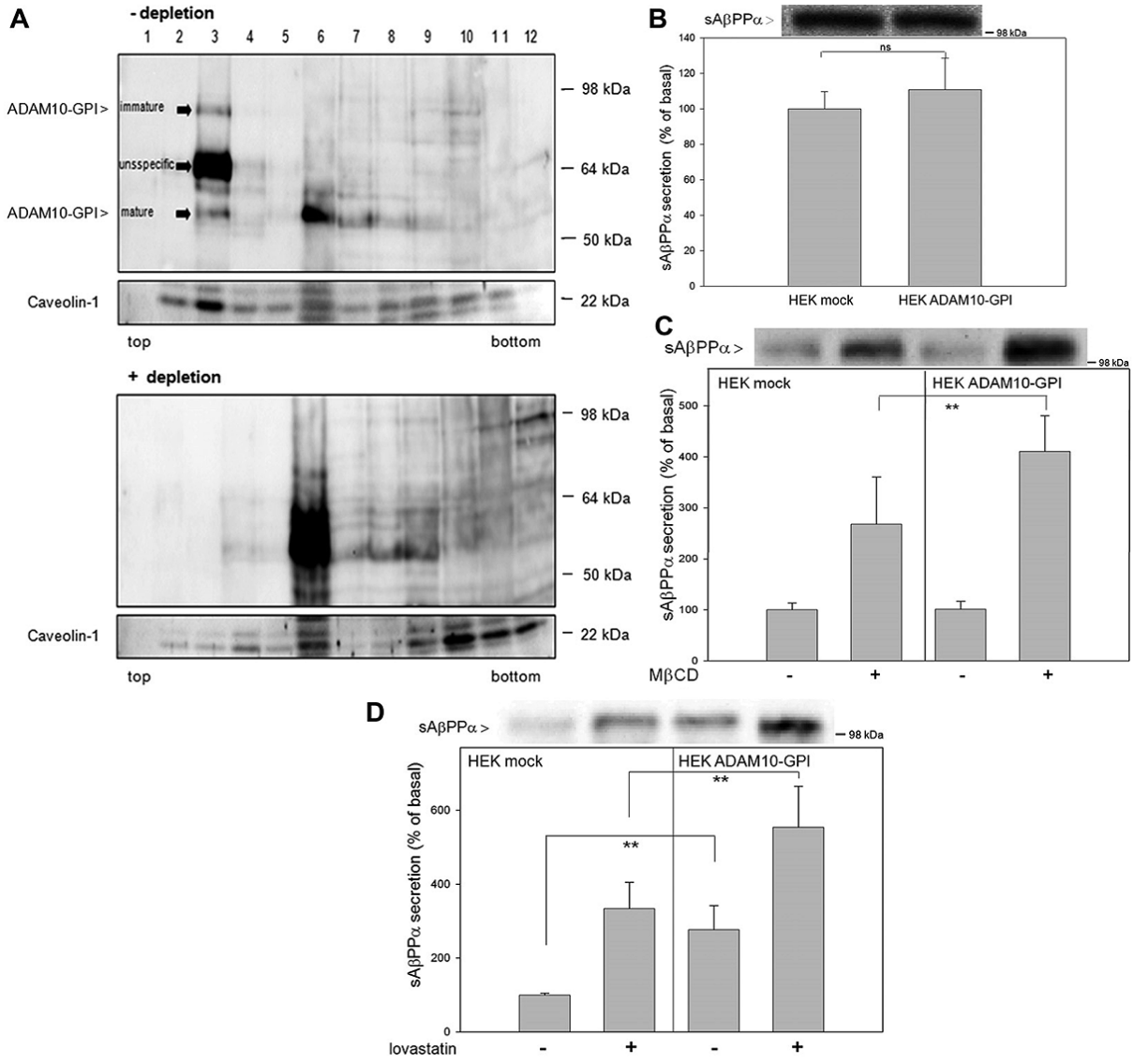

Fig. 8. Influence of a GPI anchor on the α-secretase activity of ADAM10. A) Localization of ADAM10-GPI in rafts. HEK ADAM10-GPI cells were treated for 30 min either with DMEM (- cholesterol depletion, upper part) or DMEM containing 10 mM MβCD (+ cholesterol depletion, lower part), then cells were extracted with Triton X-100. After flotation in an OptiPrep step-gradient, fractions collected from the top of the gradient (1-12), were TCA precipitated and analyzed by immunoblotting with an anti-myc antibody for ADAM10-GPI and with an anti-caveolin-1 antibody for caveolin detection. B) Secretion of sAβPPα from HEK ADAM10-GPI and control cells. Cells were grown in DMEM supplemented with 10% FCS. Determination of sAβPPα was performed as described in Fig. 2. C) Influence of acute cholesterol depletion on ADAM10-GPI α-secretase activity. HEK mock and HEK ADAM10-GPI cells were incubated in the absence (lanes 1 and 3) or in the presence of 10 mM methyl-β-cyclodextrin (MβCD) for 30 min (lanes 2 and 4). After 4 h, the medium was collected and analyzed for secreted sAβPPα as described in Fig. 2. D) Effects of lovastatin and lipid-deficient serum on sAβPPα secretion from HEK ADAM10-GPI cells. HEK mock and HEK ADAM10-GPI cells were cultured in lipid-deficient medium for 24 h in the presence of 2 μM lovastatin. The detection of sAβPPα was performed as described in Fig. 2. Values are means $\pm$ SD; one-way ANOVA/Bonferroni post hoc test.

DISCUSSION

Presented results elucidate the activation mechanism of endogenously expressed α-secretase by statins. By using zaragozic acid, a potent inhibitor of squalene synthase which blocks cholesterol synthesis but allows the synthesis of isoprenoid derivatives, we could show that statin-induced α-secretase activation is a cholesterol dependent effect.

Treatment of cells with low concentration of lo-

vastatin or with zaragozic acid resulted in a significant α-secretase activation while the activity of the β-secretase was not affected. Induced α-secretase activity was strongly inhibited by two zinc metalloproteinase inhibitors: a broad spectrum inhibitor and an inhibitor which has been shown *in vitro* to be more specific for the α-secretase ADAM10 [39]. The relevance of ADAM10 in the lovastatin-induced α-secretase activation was confirmed by applying the RNAi knockdown technology.

Our results demonstrate that the mode of action of zaragozic acid is similar to lovastatin-stimulated α-secretase activation, and that the ADAM10 α-secretase activity is regulated by the sterol pathway. In a previous study, we showed that lovastatin-induced α-secretase activation was prevented by restoration of the cholesterol level [23].

The expression analyses of cells treated with lovastatin or zaragozic acid showed a slight increase of ADAM10 on the protein level, whereas the ADAM10 mRNA level and promoter activity were not altered. The lacton form of lovastatin, which can inhibit proteasome-mediated protein degradation, was not responsible for the increased stability of the ADAM10 protein. Further studies are necessary to distinguish between increase of ADAM10 translation and ADAM10 protein stability caused by cholesterol reduction. By using GPI-anchored ADAM10, we found that the C-terminus of the α-secretase ADAM10 is probably crucial for α-secretase activation by lovastatin.

The detected maximal statin concentration in human serum is $\sim$30 nM and 10 fold lower in the brain. In our experimental conditions, 30 nM lovastatin slightly increased sAβPPα secretion. To obtain experimental conditions which allow detection of significant effects and additionally imitate physiological concentrations, a lovastatin concentration of 1 or 2 μM was used in our experiments. Although this lovastatin concentration is 30–60 fold higher than found in serum, the isoprenylation pathway was not affected in our experimental conditions. Lovastatin-induced α-secretase activation was not impaired by restoration of the isoprenylation pathway (Fig. 6A, lane 2 and 3).

Under conditions where statin treatment inhibits $\sim$50% of sterol biosynthesis, which are comparable to pharmacological conditions, no observable effects on protein isoprenylation were detected [40]. The enzymes of the non-sterol pathways have higher affinities for mevalonate-derived substrates than those of the sterol pathway. When mevalonate is limited, it is preferentially used by enzymes from the non-sterol pathways [41]. Therefore, under physiological conditions this low statin concentration probably impairs only the sterol pathway whereas protein isoprenylation remains unaffected.

Previous reports described the involvement of protein isoprenylation pathways in the α-secretase activation by statins [24,26]. Our finding, that the prevention of protein isoprenylation by a geranylgeranyltransferase inhibitor enhances the α-secretase activity confirmed these results. However, the inhibition of the isoprenylation pathway requires nearly total inhibition of HMGCoA reductase which can only be achieved by non-physiological, high statin concentrations. Our results indicate that inhibition of cholesterol biosynthesis is sufficient for α-secretase activation. This condition should be achievable by physiologically low statin concentrations or squalene synthase inhibitors.

Lipid rafts are cholesterol-enriched membrane microdomains implicated in signal transduction, protein trafficking, and proteolytic processing [42]. Statin-mediated inhibition of cholesterol biosynthesis results in the disruption of lipid rafts [43,44].

A number of studies demonstrated the significance of lipid rafts in the regulation of Aβ generation. The β-secretase (BACE) is associated with rafts but the active form of the α-secretase ADAM10 is not localized in rafts. Wild-type BACE was found in rafts prepared from a human neuroblastoma cell line (SH-SY5Y), and it was shown that addition of a GPI-anchor to BACE targets the enzyme exclusively to lipid raft domains. Expression of GPI-BACE strongly increased the secretion of both sAβPPβ and Aβ peptides [45]. We used the same approach by applying the GPI-anchor to study the influence of lipid rafts on the α-secretase activity of ADAM10. Targeting ADAM10 to lipid rafts had no influence on sAβPPα secretion, but lipid rafts destruction by cholesterol depletion caused the ability of ADAM10-GPI to act as α-secretase. Localization of ADAM10 in rafts possibly leads to separation from its substrate AβPP. This confirms previous results that the activity of the α-secretase ADAM10 inversely correlates with the amount of cellular cholesterol [23].

Statins are widely used in medical practice and are the principal therapy for hypercholesterolemia. Statin treatment significantly reduces serum cholesterol but it is not clear whether statins have a direct influence on cholesterol in the central nervous system (CNS). It was reported that treatment of human subjects for 3 months with lovastatin resulted in a decrease of Aβ peptides in serum [46], whereas most clinical studies have not confirmed the effect of statins on Aβ levels in the brain [47,

48]. Activation of the α-secretase and inhibition of the β-secretase require strong reduction of cellular cholesterol, therefore it is unlikely that physiological statin concentrations can influence the production of Aβ peptides in the brain.

An explanation for the effect of statins on slowing the development of AD may be caused by equilibrium between Aβ in blood and brain. Low serum Aβ levels may facilitate the efflux of Aβ from the brain and the uptake of Aβ into the brain would also be reduced under such conditions. Aβ peptides are not exclusively produced in the CNS; blood and peripheral tissues are also sources for Aβ.

Several studies suggest that reduction of Aβ from the systemic circulation by anti-Aβ antibody [49], soluble RAGE [50], or LRP-1 [51] may be sufficient to inhibit AD development. Recently, a large cohort study showed that statins are associated with a reduced risk of developing AD. Interestingly, the observed protective effect was independent from the lipophilicity of used statins [17]. Probably, the lowering of plasma cholesterol by statins may impair formation of neurotoxic Aβ, which may slow the progression of AD.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=334).

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