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EXPERIMENTAL USE OF SELECTED CHOLESTEROL-LOWERING DRUGS AS POTENTIAL CANDIDATES IN MODIFICATION OF THE PATHOPHYSIOLOGY OF ALZHEIMER´S DISEASE

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CONTENTS

1. ABSTRACT

Rationale: The current standard treatment of Alzheimer´s disease (AD) is represented by acetylcholinesterase (AChE) inhibitors. In the pathogenesis of AD, cholesterol is directly involved. Its blood and brain levels positively correlate with amyloid β (Aβ) − a peptide characteristic for AD and capable of increasing AChE activity. Based on these data, we may suppose that cholesterol-lowering medication such as statins and alendronate might confer protection against dementia, probably via modulation of cholesterol synthesis in the brain. The aim of the present studies was to investigate possible influence of two lipophilic statins (simvastatin and atorvastatin) and alendronate on cholesterol synthesis in selected parts of the rat central nervous system (CNS) and other parameters relevant to Alzheimer´s disease pathophysiology.

Methods: We have performed 3 similar experiments. Rats were administered simvastatin, atorvastatin, alendronate, or water. Thereafter, blood and brain parts were isolated and analyzed for cholesterol, lathosterol, Aβ 40 and 42, hydroxymethylglutaryl-coenzyme A reductase protein, catalytic concentration of acetylcholinesterase and cholesterol synthesis rate.

Results: All drugs at higher doses were able to lower cholesterol in the plasma, but none elicited an effect on total brain cholesterol. Significant reductions of lathosterol and cholesterol synthesis rate were observed after simvastatin- and atorvastatin- treatment, whereas amyloid beta and hydroxymethylglutaryl-coenzyme A reductase levels remained unaffected. Both statins elicited comparable effects on cholesterol synthesis rate irrespective of the examined brain part. In the alendronate group, the experiments brought inconsistent results concerning its influence on brain cholesterol synthesis. Both simvastatin and alendronate inhibited acetylcholinesterase activity in the frontal cortex.

Conclusions: Our studies bring additional evidence of the role of statins in the brain cholesterol synthesis and are the first to show an influence of statins and alendronate on AChE activity in the rat brain. However, our data question the relationship between amyloid beta, acetylcholinesterase activity and cholesterol synthesis. For comparison of the effects of statins in selected parts of the brain, the deuterium technique was utilized for the first time.

2. SOURHN V ČEŠTINĚ

Úvod: Současný standard v terapii Alzheimerovy demence (AD) představují inhibitory acetylcholinesterázy (AChE). V patogenezi AD hraje roli cholesterol, jehož hladiny v krvi a mozku pozitivně korelují s amyloidem β (Aβ) – proteinem charakteristickým pro AD a schopným zvyšovat aktivitu AChE. Předpokládali jsme, že léky zasahující do biosyntézy cholesterolu by mohly chránit proti demenci, pravděpodobně modulací syntézy cholesterolu v mozku. Cílem bylo proto zkoumat možný vliv lipofilních statinů a alendronátu na syntézu cholesterolu ve vybraných částech centrálního nervového systému (CNS) potkana ve vztahu k dalším parametrům relevantním pro patofyziologii AD.

Metodika: Realizovali jsme 3 obdobné experimenty, kdy jsme potkanům podávali simvastatin, atorvastatin, alendronát (v různém dávkování) nebo vodu. Následně byla izolována krev a části mozku ke stanovení hladiny cholesterolu, lathosterolu, proteinu hydroxymethylglutaryl-koenzym A reduktázy, katalytické koncentrace AChE, koncentrace Aβ40, Aβ42 a rychlosti syntézy cholesterolu (pomocí metodiky s oxidem deuteria, který byl potkanům podáván vždy od druhého dne experimentu).

Výsledky: Podávané léky byly při delším podávání schopny snížit hladinu cholesterolu v plazmě, aniž by ovlivnily celkový cholesterol v mozku. Signifikantního snížení syntézy cholesterolu (hodnoceno dle poklesu lathosterolu a rychlosti syntézy cholesterolu s použitím metodiky s oxidem deuteria) bylo dosaženo po podávání jak simvastatinu, tak atorvastatinu, přičemž jejich efekt byl závislý na podané dávce. Oba statiny vykazovaly srovnatelný vliv na rychlost syntézy cholesterolu ve všech vyšetřených částech mozku. Neprokázali jsme předpokládanou up-regulaci proteinu hydroxymethylglutaryl-koenzym A reduktázy. Také koncentrace Aβ zůstala nezměněna. Simvastatin i alendronát inhibovaly aktivitu AChE ve frontálním kortexu. Ve skupinách s alendronátem bylo při studiu jeho vlivu na syntézu cholesterolu v mozku dosaženo nejednotných výsledků.

Závěry: Potvrdili jsme vliv statinů na syntézu cholesterolu v různých částech CNS (vůbec poprvé technikou s oxidem deuteria), přičemž byla nově zjištěna závislost efektu na dávce. Nález stabilní koncentrace celkového cholesterolu v mozku po statinech poukazuje na schopnost potkana zachovávat jeho homeostázu. Ve sledovaných parametrech jsme neprokázali rozdíl v účinku atorvastatinu a simvastatinu. Přestože naše výsledky svědčí pro vliv statinů a alendronátu na aktivitu AChE, vztah mezi amyloidem beta, aktivitou AChE a syntézou cholesterolu v mozku potkanů zpochybňují.

3. ORIGINAL STUDIES

This thesis is based on the following papers which will be referred to in the text by their Roman numerals.

STUDY I.

Cibickova Lubica, Palicka Vladimir, Cibicek Norbert, Cermakova Eva, Micuda Stanislav, Bartosova Lucie, Jun Daniel: **Differential effects of statins and alendronate on cholinesterases in serum and brain of rats.** Physiol Res 2007;56(6):765-770. **IF = 2.1**

STUDY II A.

Cibickova Lubica, Hyspler Radomir, Ticha Alena, Cibicek Norbert, Palicka Vladimir, Cermakova Eva, Zadak Zdenek: **Cholesterol synthesis in central nervous system of rat is affected by simvastatin as well as by atorvastatin.** Pharmazie 2008;63:819-22. **IF = 0.67**

STUDY II B.

Cibickova Lubica, Palicka Vladimir, Hyspler Radomir, Cibicek Norbert, Cermakova Eva: **Alendronate lowers cholesterol biosynthesis in the central nervous system of rats – a preliminary study**. Physiol Res 2009;58(3). Accepted for publication. **IF = 2.1**

STUDY III.

Cibickova Lubica, Hyspler Radomir, Micuda Stanislav, Cibicek Norbert, Zivna Helena, Jun Daniel, Ticha Alena, Palicka Vladimir: **The influence of simvastatin, atorvastatin, alendronate and high cholesterol diet on acetylcholinesterase activity, amyloid beta and cholesterol synthesis in rat brain.** Steroids 2008. Accepted for publication. **IF = 2.4**

4. ABBREVIATIONS

AD - Alzheimer´s disease;

ApoE - Apolipoprotein E;

APP - amyloid precursor protein;

APPs - soluble amyloid precursor protein;

Aβ - amyloid β-peptide;

AChE - acetylcholinesterase;

BuChE - butyrylcholinesterase;

BBB - blood-brain barrier;

CNS - central nervous system;

FSR - fraction synthesis rate;

HMG CoA -3-hydroxy-3-methylglutaryl coenzyme A;

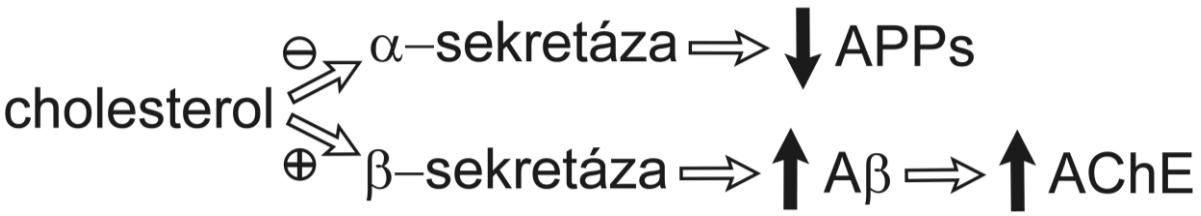
N-BPs - nitrogen-containing bisphosphonates.

5. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder with progressive cognitive impairment, personality changes and memory deficits largely attributable to deficiency in cholinergic neurotransmission. Post mortem, the unit is histopathologically characterized by an abnormal brain accumulation of amyloid β-peptide (Aβ). Current therapeutic strategies for AD focus on cognitive deficit alleviation via direct AChE inhibition (Jirák, Koukolík 2004). Even though traditional medications like donepezil, rivastigmin or galantamin are at present a "gold standard" in this aspect, they are indicated as symptomatic drugs, may be less tolerable and representing a considerable financial burden. Questions arise, if present evidence points to any alternative/additional therapeutic approach that might intervene in the pathogenesis of the disease and concurrently enhance tolerability and/or reduce costs associated with the treatment of AD.

As alternatives to reduce symptoms of AD and slow the progression of the disease, several different classes of medicaments have been suggested, e.g. Aβ vaccination, metal chelators, anti-inflammatory drugs, and cholesterol-lowering drugs. Some, but not all (Rea *et al.* 2005; Zandi *et al.* 2005) of recent epidemiological reports indicate that cholesterol-lowering medicine such as statins might confer protection against dementia (Rockwood *et al*. 2002; Zamrini *et al.* 2004). The idea of utilizing cholesterol-modifying agents comes from experimental studies indicating a significant role of cholesterol in the pathophysiology of AD. Higher serum levels of cholesterol elevate Aβ and facilitate its deposition into plaques, which are of importance for the development of the disease (Sparks 1996). In addition, cholesterol inhibits α-secretase and hence hinders the production of neuroprotective soluble amyloid precursor protein (APPs) (Bergmann 2000). Moreover, Aβ has recently been documented to increase acetylcholinesterase (AChE) activity *in vitro* (Hu *et al.* 2003) (Fig. 1). However, the exact mechanism of their action on AD has not been clarified yet.

Fig. 1: Relationship between cholesterol, Aβ and AChE



6. AIMS OF THE STUDY

Based on the published data, I have decided to examine selected cholesterol-lowering drugs as potential candidates in modification of the pathophysiology of Alzheimer´s dementia. I tested the hypothesis that cholesterol-lowering drugs have an influence on cholesterol metabolism, cholinesterase activities and Aβ in the rat brain. For this purpose I utilized selected representatives from two drug groups − statins (simvastatin and atorvastatin) and bisphosphonates (alendronate).

7. MATERIALS AND METHODS

7.1. Animals

Adult male rats of Wistar strain (240-280 g) were obtained from Biotest, Konárovice, Czech Republic. Animals were housed in the animal quarters for at least seven days prior to experiments under controlled environmental conditions (22–24°C, 40–60% relative humidity, air exchange 12–14 times h^{-1} and 12–hour light–dark cycle periods). The rats had free access to standard laboratory rat chow pellets (ST 1-TOP, Velas, Prague, Czech Republic) except for 16–18 h before and 0.5 h after the experiment. Tap water was provided *ad libitum*. Drugs were administered by oral route every day between 9.00 and 11.00 a.m. for 7 days (*Study I*) or 9 days (*Study II*) or 15 days (*Study III*). All animals received care in accordance with the guidelines set by the institutional Animal Use and Care Committee of the Charles University in Prague, Czech Republic. All experimental procedures were approved by the Committee for Protection of Animals against Cruelty (Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic).

7.2. Experimental protocol

The animals were randomly divided into four groups, eight subjects in each. The first (sham) group served as control and received the vehicle (water) only, the second group was administered atorvastatin (50 mg/kg, Pfizer, Gorecke AG Freiburg, Germany), the third ingested simvastatin (50 mg/kg, IVAX Pharmaceuticals, Czech Republic), and the fourth was exposed to alendronate (3 mg/kg, MSD, Merck Sharp & Dohme B.V., Netherlands). The used

dosage was based on published data. On the last day of experiment, 1 h after the last drug application (respecting c_{max}), the animals were sacrificed by exsanguination (blood withdrawal) from abdominal aorta under ether anesthesia. The brains were immediately exteriorized and the studied brain tissues (basal ganglia, septum, frontal cortex, and hippocampus) isolated. The specimens were stored in plastic vials at –20 °C until analysis.

Study II was similar to the first. There were only some differences:

Tap water was provided *ad libitum* until the second day of experiment. The second day rats received a loading dose of deuterated water (35 mL/kg 99% enriched ^2H_2O) and then had free access to drinking water enriched with 10% ^2H_2O (Diraison et al. 1996). The dosage of statins was lowered: atorvastatin (10 mg/kg) and simvastatin (10 mg/kg). The lowering of statin dosage was based on pre-experimental data, where higher dosage (50 mg/kg) produced immeasurable inhibition of cholesterol synthesis. The brains were not divided in four parts, but small samples from the basal ganglia, frontal lobe, hippocampus, and spinal cord were isolated.

Study III was similar to the second with these differences:

Animals were randomly divided into 8 groups, 6 subjects in each. The first (sham) group received the vehicle only (*aqua pro injectione*), the second was administered simvastatin (10mg/kg b.wt.), the third ingested atorvastatin (10 mg/kg b.wt.), the forth simvastatin (20 mg/kg b.wt), the fifth atorvastatin (20 mg/kg b.wt.), the sixth alendronate (3 mg/kg b.wt.), and the seventh also alendronate (6 mg/kg b.wt.). The last group received only *aqua pro injectione*, but was fed with a cholesterol-enriched diet (2% cholesterol, Albert Weber – SEMED, Prague, Czech Republic).

7.3. Biochemical analyses

7.3.1 Plasma cholesterol

For determination of cholesterol in the blood, plasma samples were analyzed using a routine analyzer Modular Roche (Roche diagnostics, Mannheim, Germany) according to the manufacturer´s instructions.

7.3.2. Determination of acetylcholinesterase and butyrylcholinesterase activities in plasma

Erythrocytes were obtained from plasma by centrifugation (10 min at 3000 RPM at laboratory temperature). The activities of AChE and BuChE were determined using the modified method of Ellman *et al.* (1961). Briefly, to 0.4 ml of 5,5'-dithiobis-2-nitrobenzooic acid (DTNB) in 0.2 ml 1,1,1-tris-(hydroxymethyl)aminomethan (TRIS)-HCl buffer (pH 7.6) 100 µl of

hemolyzate (50 µl erythrocytes and 950 µl water) of erythrocytes (or of plasma – for measurement of BuChE activity) and 1.30 ml TRIS-HCl buffer was added. The reaction was initiated by addition of 0.2 ml of the substrate−acetylthiocholine (for measurement of AChE activity) or butyrylthiocholine (for measurement of BuChE activity). The absorbance was measured at 412 nm for 5 min using a spectrophotometer (UVIKON 942). Solutions of different cystein concentrations (added instead of hemolyzate) were prepared for calibration. Each measurement was repeated three times. All measurements were conducted under standard laboratory temperature.

7.3.3 Brain cholesterol, lathosterol and cholesterol synthesis rate determination

Brain hemispheres were homogenized using IKA T10 basic, Ultra-Turrax homogenizer (IKA-werke, Germany) and extracted according the method of Bligh and Dyer (Bligh and Dyer, 1959). Briefly, tissue samples were mixed with methanol: water solution (2:0.8), enriched with internal standards and extracted to chloroform using a Stuart rotator (Barloworld Scientific, Stone, UK). The chloroform layer was separated, evaporated to dryness and cholesterol was derivatized using BSTFA (bis-trimethylsislyl-trifluoroacetamide, Supelco, Bellefonte, USA) solution in pyridine (1:1) for one hour at 80°C. The mixture containing cholesterol and lathosterol trimethylsilyl ethers was analyzed. Tissue concentrations of cholesterol and lathosterol were determined using GC 8000 system (Fisons instruments, UK) equipped with a flame ionisation detector. The compounds of interest were quantified using internal standard method (alpha-cholestane for cholesterol and stigmastanol for lathosterol). Results are expressed as molar concentration per gram of wet tissue. Analysis of cholesterol synthesis rate was performed on GC-MS system (Perkin-Elmer, Norwalk, USA) operating in electron ionization mode. The injector temperature was set to 300 °C, slit ratio 1:10, oven 320 °C isothermally, ionization source 280 °C. The ions m/z 368.6, 369.6 and 370.6 were recorded, isotope excess and fractional synthesis rate were calculated according to Diraison (Diraison et al. 1997). Briefly, enrichments were calculated from the observed spectral intensities of ions mentioned above. After matrix correction, values were converted into molar excess (the ratio of molecules having incorporated one or two excess deuterium atoms, mi/m0+m+1+m+2). Then, the average number of deuterium atoms incorporated was calculated IEobs = m+1+2m+2 and compared with the maximum number of deuterium atoms (twenty seven) that can be incorporated into cholesterol molecule at given enrichment of plasma water (p) IEexp = 27 x p. The deuterium oxide enrichment was determined from

plasma as described previously (Yang *et al.* 1998) using hydrogen atom exchange between water and acetone in alkaline solution. Results are expressed as the fractional synthesis rate (FSR = IEobs/IEexp) representing a fraction of cholesterol content in tissue, which was synthesized during tracer application period (14 days). This application period is suitable for tissues with a low cholesterol synthesis rate, but unfortunately does not enable the determination of synthesis rate in tissues with fast synthesis rate (e.g. liver) due to tracer cycling.

7.3.4 Expression of HMG-CoA reductase in the brain

The protein expression of HMG-CoA reductase in the brain was performed by immunoblot analysis. Samples were prepared by lysis in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SCS), 50 nM Tris-HCl, pH 8.0; Sigma-Aldrich) in the presence of protease inhibitors (1 mg/ml aprotinin, and 1 mM PMSF). Lysates were passed through a 21-gauge needle to shear the DNA and consequently incubated on ice for 30-60 minutes. Nonsolubilized debris was pelletized at 10,000 g for 10 minutes. The supernatant was collected, aliquoted, and stored at -80°C until use. Protein concentrations of cell lysates were determined by bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL).

Immunoblot analysis: Lyzates (70 ug protein/well) were incubates with sample buffer at 37°C for 30 minutes and separated on a 7.5% polyacrylamide gel. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), it was blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS). The membrane was incubated with primary antibody (1:500) for 1 h, washed, and incubated for 1 h with a peroxidase-conjugated secondary antibody (1:1.000). After washing with TTBS, the membranes were developed using an enhanced chemiluminescent reagent (GE Healthcare, ague, CZ) and subjected to autoluminography for 1-5 min. The immunoreactive bands on the exposed films were scanned with a densitometer ScanMaker i900 (UMAX, Prague, CZ) and semiquantified using a QuantityOne imaging software (Bio-Rad). Equal loading of proteins onto gel was confirmed by immunodetection of beta-actin.

7.3.5 Determination of acetylcholinesterase activity in the brain

The samples of the brain hemispheres were homogenized in distilled water using a homogeniser (Ultra Turrax T18) in a ratio 1:10 (w/w). The activity of AChE in the homogenate was determined using a modified method of Ellman *et al.* (Ellman *et al.* 1961).

Briefly, to 0.8 ml of 5,5'-dithiobis-2-nitrobenzooic acid (DTNB) in 1,1,1-tris-(hydroxymethyl)aminomethan (TRIS)-HCl buffer (0.1 M, pH 7.6) 0.1 ml of homogenised brain and 0.9 ml of TRIS-HCl buffer was added. The reaction was initiated by addition of 0.2 ml of AChE substrate−acetylthiocholine. The final concentration of DTNB and acetylthiocholine in the mixture was 1 mM. The absorbance was measured in a 1 cm cuvette at 412 nm for 1 min using a spectrophotometer (Helios Alfa). Solutions of different cystein concentrations (added instead of brain homogenate) were prepared for calibration. Each measurement was repeated three times. All measurements were conducted under standard laboratory temperature (25 °C).

7.3.6 Analysis of Aβ 1-40 and Aβ 1-42 in brain

The samples were prepared according to Borchelt *et al.* (Borchelt *et al.* 1996). Briefly, approximately 150 mg of brain hemisphere was dunce homogenized in 1 ml of 70 % formic acid. Homogenates were centrifuged at 100,000 x g for 1 h to remove particulate material. The infranatant was recovered and neutralized with a 20-fold dilution in 1 M Tris base. The analyses were performed using commercially available ELISA Kits (Human/Rat β Amyloid (40) ELISA Kit Wako II and Human/Rat β Amyloid (42) ELISA Kit Wako, High-Sensitive, Wako Chemicals GmbH, Germany) in compliance with the manufacturer´s instructions. The values obtained were adjusted for sample dilution and converted to pmol/g wet weight tissue.

7.4. Statistical analyses

The data were processed by the programs NCSS 2004 and Statistica. Normality was tested using Kolmogorov-Smirnov test. For statistical evaluation, Analysis Of Variance with *post hoc* Fisher's LSD Multiple-Comparison Test (between-group comparisons), Kruskal-Wallis Test and unpaired t-tests were applied. The chosen level of significance was α=0.05.

8. RESULTS

8.1. Levels of sterols in plasma and the brain

We investigated plasma levels of cholesterol in *Study II* and *III*, not in *Study I*. In *Study IIa* treatment neither with simvastatin (10 mg/kg) nor with atorvastatin (10 mg/kg) for 9 days produced any decrease in plasma cholesterol (p=0.31) (for details see *Table 1* in *Study IIa*). Also treatment with alendronate for 9 days did not produce any change in plasma cholesterol (1.37, 1.02–2.86 for alendronate vs. 1.24, 0.98–2.50 for controls; p=0.44, results are expressed as median, minimum–maximum).

In *Study III*, both simvastatin and atorvastatin at a higher dose (20 mg/kg) and longer duration of treatment (15 days) influenced plasma and brain sterols. In animals treated with simvastatin (20 mg/g), plasma concentrations of cholesterol were reduced by 27 % ($P < 0.001$) and those of lathosterol in the brain were reduced by 19 % ($P < 0.01$). The ratio of brain lathosterol to cholesterol was lowered by 26 % ($P < 0.001$). Treatment with atorvastatin (20 mg/g) also revealed a 20 % ($P < 0.05$) reduction of plasma concentrations of cholesterol and a 12% ($P < 0.05$) reduction of brain lathosterol, while the ratio of brain lathosterol to cholesterol was lowered by 26 % ($P < 0.01$). The mean brain cholesterol level was not different in any group compared to controls ($P = 0.16$) (for details see *Table 1* in *Study III*).

8.2. Cholesterol synthesis rate and HMG-CoA reductase expression in the brain

Brain cholesterol synthesis in the control (sham) group was compared with those measured in the atorvastatin, simvastatin alendronate groups. Treatment with all three drugs, for the dose and time investigated, significantly decreased cholesterol synthesis in all investigated CNS parts (basal ganglia, hippocampus, frontal lobe, and spinal cord) in *Study II* (for details see *Table 2* in *Study IIa and Table 1 in Study IIb*) and similarly in the brain hemisphere homogenate in *Study III* (*Figure 1* in *Study III*). The higher dose of statins elicited a greater lowering of FSR than the lower dose, 77 % for simvastatin ($P < 0.001$) and also 77 % for atorvastatin ($P < 0.01$). The same dose-dependent effect was observed on the brain lathosterol/ cholesterol ratio (*Table 1* in *Study III*). However, statins elicited comparable effects on cholesterol synthesis irrespective of the examined tissue.

However, none of the used treatments has manifested with a statistically significant effect on HMG-CoA reductase expression (one-sample t-test of a difference from 100 %, *Figure 2* and *Table 2* in *Study III*).

8.3. Influence on cholinesterases

8.3.1. Effect on AChE and BuChE in blood

In *Study I*, we investigated a possible impact of statins and alendronate on acetylcholinesterase and butyrylcholinesterase activities in blood (erythrocytes) and serum, respectively. One hour after the last administration of drugs, trunk blood was collected and the enzymatic activities measured. Comparison of data from the control group with respective values from the drug-treated animals did not show any statistically significant change in the activity of neither AChE nor BuChE (see *Figure 1* in *Study I*).

8.3.2. Effect on AChE in the brain

AChE activity in the control (sham) group was compared with those measured in atorvastatin (50 mg/kg), simvastatin (50 mg/kg) and alendronate (3 mg/kg) groups in *Study I*. Seven-day administration of these agents had no statistically significant effect on AChE activity in the septum, basal ganglia or hippocampus (see *Figure 2* in *Study I*). In contrast, simvastatin and alendronate significantly decreased AChE activity in the frontal cortex ($P < 0.05$, *Figure 2* in *Study I*).

In *Study III*, none of the used treatments (atorvastatin 10 or 20 mg/kg, simvastatin 10 or 20 mg/kg and alendronate 3 or 6 mg/kg) has expressed a statistically significant effect on acetylcholinesterase activity in brain hemisphere homogenate ($P = 0.18$) (see *Table 2* in *Study III*).

8.4. Concentration of amyloid beta in the brain

None of the used treatments (atorvastatin 10 or 20 mg/kg, simvastatin 10 or 20 mg/kg and alendronate 3 or 6 mg/kg) has expressed a statistically significant effect on brain Aβ(40) ($P = 0.76$) and Aβ(42) ($P = 0.38$) concentrations (see *Table 2* in *Study III*).

9. DISCUSSION

9.1. Effects of statins

9.1.1. Biomarkers of cholesterol synthesis in the brain

Nearly all input of cholesterol into the CNS comes from *in situ* synthesis. Total cholesterol, 24S-hydroxycholesterol, lathosterol, and 27-hydroxycholesterol are used as biomarkers for cholesterol metabolism monitoring. Cholesterol 24-hydroxylase (CYP46a1) in the brain is capable of converting cholesterol to *24S-hydroxycholesterol* (cerebrosterol), which can cross the BBB, enters the plasma and hence can be used as a marker for cholesterol elimination (Dietschy and Turley 2001). Lathosterol is a cholesterol precursor and its ratio to cholesterol is believed to be a marker for cholesterol endogenous synthesis (Lutjohann et al. 2004).

However, to measure the absolute rate of sterol synthesis *in vivo*, quantitating the rates of incorporation of either ^{2}H or ^{3}H atom from [^{2}H] water of [^{3}H] water into cholesterol molecule is recommended (Dietschy and Turley 2001). We have used (in *Study II and III*) $^{2}H_2O$ which is a safe, nonradioactive tracer and can be administered *per os*.

9.1.2. Influence on cholesterol synthesis

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which controls the key step in cholesterol biosynthesis. Among the widely prescribed statins, simvastatin (4.68), atorvastatin (4.06), lovastatin (4.27) and fluvastatin (3.24) are lipophilic, while pravastatin (-0.22) is regarded as a hydrophilic compound (in brackets: logarithm of the partition coefficient octanol/water, Corsini *et al.* 1999, Schachter 2005).

Some experimental trials have investigated the effects of different statins on biomarkers of cholesterol synthesis and elimination (their results are summarized in *Table 3, Study IIa*). Generally speaking, statins have the ability to suppress cholesterol synthesis in the brain (i.e. lower lathosterol levels). Also in our studies, the administration of both simvastatin and atorvastatin suppressed cholesterol synthesis in the rat brain as evaluated by lathosterol, lathosterol/cholesterol ratio (*Study III*) and cholesterol FSR (*Study IIa, Study III*). The decrease in cholesterol FSR and lathosterol/cholesterol ratio was dose-dependent, which confirms the relationship between statins and brain cholesterol synthesis. Concerning FSR, no statistically relevant difference between the corresponding simvastatin and atorvastatin groups was observed (i.e. the drugs had similar potency to produce a reduction of this parameter).

This finding is in agreement with previous reports, where 3-week administration of simvastatin vs. pravastatin resulted in comparable effects with respect to lathosterol suppression (Lutjohann *et al.* 2004; Johnson-Anua *et al.* 2005). Nonetheless, the rat brain was found to have a remarkable capacity to maintain cholesterol homeostasis in spite of considerable changes in FSR and also in the levels of circulating cholesterol, i.e. neither brain cholesterol FSR, nor reduction/elevation of plasma cholesterol had a significant effect on total brain cholesterol levels, a finding consonant with studies on guinea pigs (Lutjohann *et al.* 2004) or transgenic mice (Petanceska *et al.* 2002). This phenomenon may be explained either by suppressed cholesterol elimination (Lutjohann *et al.* 2004) or potentiated synthesis (manifested as significant up-regulation of HMG-CoA mRNA expression and activity in simvastatin-treated animals) in the brain tissue (Thelen *et al.* 2006, Franke *et al.* 2006). The latter may presumably represent a compensatory mechanism responsible for counteracting the inhibitory effect of high-dose statin treatment on brain cholesterol-synthesis (Thelen *et al.* 2006). Since we neither determined the marker of cholesterol elimination (24S-OH-cholesterol) nor HMG-CoA reductase activity, the stable levels of HMG-CoA reductase protein do not support the notion of its compensatory up-regulation and rather suggest suppressed cholesterol elimination. It also may be speculated that the failure to confirm significant compensatory elevations of HMG-CoA reductase protein in this study may be attributed to considerably (10-times) lower doses of drugs used. Nevertheless, this is the first study to measure total brain HMG-CoA reductase protein in statin-treated rats (*Study III*).

In this aspect it would be interesting to see if the effect on brain cholesterol metabolism is dependent on the grade of lipophilicity (which determines the drug´s BBB transport). Some authors suggest that there is a slight difference between the effects of lipophilic (simvastatin) and hydrophilic (pravastatin) statins (in favor of the former) on brain cholesterol synthesis (Thelen *et al.* 2006). Moreover, Johnson-Anuna *et al.* demonstrate that although cholesterol levels fail to differ significantly between pravastatin and simvastatin-treated mice, the levels of statins in the cerebral cortex do reflect their hydrophobicity producing a greater reduction in cholesterol synthesis in simvastatin vs. pravastatin group (Johnson-Anuna *et al.* 2005). On the other hand, Lutjohann *et al.* suggest that brain cholesterol synthesis in guinea pigs is influenced by simvastatin as well as by pravastatin (Lutjohann *et al*. 2004). We examined the effects of simvastatin vs. atorvastatin using ten-times lower dose and somewhat longer duration of treatment (9 days vs. 3 days) than Thelen *et al.* (Thelen *et al.* 2006). To a large extent, the lack of variance between the two drugs used in the present study in terms of their effect on brain cholesterol synthesis may be attributed to a relatively small difference in

lipophilicity (atorvastatin being less lipophilic than simvastatin), and/or the ability of both statins to cross the BBB, although the transport mechanism and permeation rate may differ (Tsuji *et al.* 1993). The reason is the different form of the administered drug–atorvastatin, fluvastatin and pravastatin are normally administered in an active hydroxy-acid form (Malhotra and Goa, 2001), but simvastatin as an inactive lactone prodrug of the active hydroxy-acid form. HMG-CoA reductase inhibitors of lactone form are then able to simply diffuse through the BBB, whereas those having an acid form are transported via a carrier-mediated transport system (Tsuji *et al.* 1993). This may help explain why atorvastatin is sometimes classified among hydrophilic compounds (Sparks *et al.* 2002) not crossing the BBB to any significant extent (Knopp 1999) in contrast to other studies, which expect considerable penetration of atorvastatin across the BBB (Kishi *et al.* 2008, Chen *et al.* 2008, Tanaka *et al.* 2007). As far as hydrophilic substances are concerned, the findings of their BBB permeation were supported also by microdialysis studies showing higher probability for active transport across the BBB for more drugs than expected. Hence, earlier assumptions that hydrophilic drugs have a slow (passive) equilibration across the BBB due to low permeability are largely questioned (de Lange *et al.* 2000). Therefore it seems that more lipophilic as well as more hydrophilic statins may directly lower brain cholesterol synthesis through local influence on brain tissue.

The effect of statins on brain cholesterol synthesis can also be considered indirect (via lowering plasma cholesterol). This point of view is advocated by Sparks *et al.* who hypothesize that reducing cholesterol in the blood be the safest way to decrease brain cholesterol and that direct inhibition of cholesterol synthesis within the CNS be ill-advised. By reducing cholesterol levels in the circulation, brain cholesterol will be lowered passively and safely (Sparks *et al.* 2002). This idea presupposes that brain and plasma cholesterols are exchangeable. Although the assumption of exchangeability has been challenged by some authors (for review see Dietschy and Turley 2004), others support the concept of low, but significant transport of cholesterol from the circulation into the brain (Lutjohann *et al.* 2004, Serougne *et al.* 1975). This discrepancy may be accounted for by different methods used for the measurement of cholesterol flux and by the rates of cholesterol synthesis being too low to be detected by current methods (Dietschy and Turley 2001). In the present experiment we have demonstrated that both statins are able to lower brain cholesterol synthesis also without altering plasma cholesterol (see *Study IIa*), for which reason we adhere to the concept of local effect of statins on brain cholesterol synthesis. In the future, a direct measurement of the drugs in the brain tissue will bring valuable information.

9.1.3. Cholesterol synthesis in various parts of the CNS

The above mentioned studies have not investigated cholesterol and/or its synthesis in different parts of the animal brain. Some of them have examined the cerebral cortex (Johnson-Anua *et al.* 2005; Petanceska *et al.* 2001), the others whole brain homogenates (Lutjohann *et al.* 2004; Thelen *et al.* 2006 Mar). In our second study, we have taken samples from three parts of the rat brain (basal ganglia, hippocampus, and frontal cortex) and the spinal cord. Cholesterol synthesis rate was the lowest in the spinal cord both in the simvastatin and atorvastatin groups (see *Study IIa*).

9.1.4. Influence on acetylcholinesterase and amyloid beta

One reason why statins and their influence on brain cholesterol metabolism are recently studied is that cholesterol was shown to be directly involved in the pathogenesis of Alzheimer's disease (AD). Cholesterol level in the brain and blood was found to positively correlate with the amount of Aβ, the characteristic AD protein (Kuo *et al.* 1998, Arvanitakis *et al.* 2000). Following statin treatment, some experimental studies documented decreased cell culture Aβ formation (Buxbaum *et al.* 2001, Fassbender *et al.* 2001), lower Aβ levels in the cerebrospinal fluid and brain homogenate of guinea pigs (Fassbender *et al.* 2001) and in the brain of transgenic mice with Alzheimer´s amyloidosis (Petanceska *et al.* 2002). On the contrary, human trials using statins lack significant changes in this pathological protein either in plasma or cerebrospinal fluid (Hoglund *et al.* 2004, Hoglund *et al.* 2005, Sjogren *et al.* 2003). These discrepancies may, to a large extent, be due to pharmacological doses of the drugs administered in humans, which may have proved insufficient to produce effects comparable to studies involving animals or cell cultures. In the present experimental study (*Study III*), 10-times lower doses (than those usually applied in experiments) were used. On one hand, this brought the given dosage closer to human medicine, but on the other hand, it may also account for our failure to observe significant alteration in brain Aβ levels.

Current therapeutic strategies for AD focus on inhibition of acetylcholinesterase (AChE), but the data concerning possible relationship between AChE and Aβ are very limited. Following addition of Aβ, Hu *et al.* documented elevated AChE expression in cultured cells (Hu *et al.* 2003). In our first study, we have also shown a decreased AChE activity in the brains of rats pretreated with simvastatin (50 mg/kg) (see *Study I*). A tendency towards lower AChE activity after statin treatment was observed also in the third experiment, although this trend failed to reach statistical significance (see *Study III*). A possible explanation for this inconsistency could lie in the fact that in the present study AChE activity was determined in

the whole hemisphere and not selectively in the frontal cortex and also in the lower doses of statins used for treatment (10 and 20 mg/kg vs. 50 mg/kg) in *Study III*. However, we failed to demonstrate the relationships between Aβ and AChE activity and between Aβ and cholesterol. Despite a marked reduction in cholesterol synthesis, we did not observe any change in Aβ levels. It is possible that statins may affect cholinergic system in the brain under the terms of their pleiotropic effects without any relationship to cholesterol.

9.2 Effects of alendronate

As a molecular target of bisphosphonates in the cholesterol biosynthesis pathway, farnesyl diphosphate synthase (= isopentenyl transferase) was identified (Rezka and Rodan 2004). Concerning their potency to inhibit cholesterol biosynthesis, Canniga *et al*. demonstrated the ability of supratherapeutic doses of etidronate to lower human serum cholesterol and total lipid levels (Canigga *et al.* 1974). In addition, pamidronate induced a weak decrease in total cholesterol and shifted cholesterol from the low-density-lipoprotein to high-density-lipoprotein fraction in patients with Paget´s bone disease (Montagnini *et al.* 2003). However, in ovariectomized rats, 3mg/kg b.wt. alendronate failed to produce a significant effect on blood cholesterol (Frolik *et al.* 1996). Our studies showed that when normal rats are used, this dose of alendronate is able to produce no (after 9 days, *Study IIb*) or only a weak (after 15 days, *Study III*) decrease in total plasma cholesterol.

As far as the brain is concerned, in our second study we have found a significant decrease in cholesterol synthesis rate in the hippocampus, frontal lobe, and spinal cord (*Table 1, Study IIb*), whereas the findings of our third study did not indicate the ability of alendronate to decrease tissue cholesterol or suppress its synthesis in the whole hemisphere (as seen in stable cholesterol, HMG-CoA reductase and cholesterol synthesis rate).

Unaware of a study describing an influence of bisphosphonates on cholinesterases, we hypothesized that cholesterol-lowering potency of these drugs might−like statins−prove beneficial in reduction of cholinesterases activities in blood and/or the brain. However, alendronate was not able to significantly decrease cholinesterase activity in serum (*Study I*).

Like simvastatin, alendronate significantly decreased the activity of AChE in the frontal cortex, a change not seen in other studied parts of the brain (*Study I*). A tendency towards lower AChE activity after alendronate treatment was observed also in the third experiment, although this trend failed to reach statistical significance (*Study III*). Moreover, no effect of alendronate on Aβ-40 or 42 was observed (*Study III*).

A possible explanation for this inconsistency could lie in the fact that instead of selective measurements in the frontal cortex, whole hemispheres were utilized in the present study. Direct determinations of alendronate bioavailability in the brain would bring valuable information to the discussion in the future.

9.3 Effects of high-cholesterol feeding

The exposure of rats to 2 % cholesterol diet, as realized in the present study, presented with a trend towards increased plasma cholesterol, whereas total brain cholesterol, lathosterol, brain cholesterol FSR as well as HMG-CoA protein remained unchanged as did Aβ and AChE activity. As to HMG-CoA reductase, similar results were obtained in its rat brain mRNA levels following 1% cholesterol diet (Jurevics *et al.* 2000). In contrast, higher amounts of cholesterol in the diet (1500 mg/day, which approximates 6-7 % cholesterol diet) led to markedly higher serum cholesterol and a significant decrease in brain cholesterol synthesis (as indicated by reduced brain lathosterol), but still did not affect total brain cholesterol in guinea pigs (Lutjohann *et al.* 2004). Notably, a diet containing 5 % cholesterol resulted in significantly increased levels of Aβ peptides in the CNS of transgenic mice (Refolo *et al.* 2000). These findings suggest that high-cholesterol diet may modify brain cholesterol synthesis and Aβ, but the effects are likely to be dose-dependent.

10. CONCLUSIONS

According to our best knowledge, the present experiments (*Study I* and *III*) are the first to show an influence of statins and alendronate on AChE activity in the rat brain. Moreover, our second study is the first experimental study comparing the influence of statins and alendronate on cholesterol synthesis in different parts of the rat brain using the incorporation of deuterium from deuterated water (*Study IIa* and *IIb*).

In our experiments, we confirmed the impact of statins on brain cholesterol synthesis using lathosterol, HMG-CoA reductase levels and the incorporation of deuterium from deuterated water (*Study III*). In comparison with prior studies we have used much lower doses of drugs, whereas the dose 20 mg/kg of statin for 15 days decreased plasma cholesterol and mimicked the effect described in humans.

The data concerning the effect of alendronate on cholesterol synthesis are inconsistent and need to be reevaluated using higher doses of alendronate.

Data from the third study question the relationship between Aβ, AChE and brain cholesterol synthesis (*Study III*) and that is why further experiments to examine the mechanism by which statins may reduce the risk of developing AD are needed.

Nowadays statins and alendronate are widely used and our results support the idea of their additional benefit. If the outcomes of our pilot experiments find support in other trials, they might have clinical implications in the therapy of Alzheimer's disease.

11. FUTURE PERSPECTIVES

A large number of articles published annually about the epidemiology, pathophysiology and treatment of Alzheimer´s disease on Pubmed indicate a great scientific interest in these issues. Concerning new approaches to treatment, attention is focused on statins and their role in the pathophysiology of AD in general and brain cholesterol metabolism in particular. The present thesis added its part to widening our knowledge of possibilities and limitations of the use of statins in this respect. Hence, a few lines of future research emerge – a deeper study of the mechanisms whereby statins act in the CNS (including the effects on cholesterol elimination and HMG-CoA reductase activity) with parallel determination of the drugs directly in the brain tissue (possibly with the aid of in situ microdialysis). The influence of alendronate on the CNS also remains to be elucidated.

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13. PUBLISHED ARTICLES AND ABSTRACTS RELATED TO MY PHD THESIS

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14. FULLTEXTS OF ORIGINAL ARTICLES