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Brain cholesterol synthesis in mice is affected by high dose of simvastatin but not of pravastatin

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d) Abbreviations 24S-OH-Chol, 24(S)-hydroxycholesterol; ABCA1, ATP-binding cassette transporter A1; AD, Alzheimer disease; ApoE, Apolipoprotein E; BBB, blood-brain barrier; CYP46A1, cholesterol 24(S)-hydroxylase; GC-MS, gas chromatography-mass spectrometry; HMG-CoAR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HPRT, hypoxanthine guanine phosphoribosyl transferase; RT-PCR, reverse transcriptase polymerase chain reaction.

e) neuropharmacology

ABSTRACT

On a global scale, there is an increasing tendency for a more aggressive treatment of hypercholesterolemia. Minor effects of statins on brain cholesterol metabolism have been reported in some *in vivo* animal studies, and it seems that this is due to a local effect of the drug. We treated male mice of the inbred strain C57/BL6 with a high daily dose of lipophilic simvastatin (100 mg/kg body weight) or hydrophilic pravastatin (200 mg/kg b.w.) or vehicle (controls) by oral gavage for three days. In order to compare the impact of both statins on brain cholesterol synthesis and degradation, levels of cholesterol, its precursor lathosterol, its brain metabolite 24(S)-hydroxycholesterol, as well as statin concentrations were determined in whole brain lipid extracts using mass spectrometry. The expression of HMG-CoA reductase mRNA and of other target genes were evaluated using real-time reverse-transcriptase PCR. In addition, analysis of liver and serum samples was performed.

Similar levels of simvastatin and pravastatin were detected in whole brain homogenates. Cholesterol content in the brain, liver, and serum was not affected by high-dose statin treatment. While brain cholesterol precursor levels were reduced in simvastatin treated animals only, no effect was observed on the formation of the brain cholesterol metabolite, 24(S)-hydroxycholesterol. PCR analysis revealed that mRNA expression of HMG-CoA reductase and ABCA1 in the brain was significantly upregulated in simvastatin treated animals compared with pravastatin treated or control animals. We conclude that, under present experimental conditions, brain cholesterol synthesis is significantly affected by short-term treatment with high doses of lipophilic simvastatin, while whole brain cholesterol turnover is not disturbed.

Introduction

Statins are first-line therapy in the treatment of cholesterol-induced atherosclerotic-cardiovascular diseases (Grundy et al., 2004). They are structural analogues of mevalonate and affect, as 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMG-CoAR) inhibitors, the rate limiting step in the cholesterol biosynthesis cascade. They inhibit conversion of HMG-CoA to mevalonate by competitive blocking of the responsible enzyme, HMG-CoAR. The endogenous cholesterol synthesis in the liver is remarkably reduced and consequently, levels of circulating LDL-cholesterol are decreased due to the increasing number of LDL-receptors on cell surfaces (Brown and Goldstein, 1981).

Within the last years plasma cholesterol has been linked to Alzheimer disease (AD) pathology and epidemiological data suggest that statin administration could be of benefit in decelerating the incidence of AD (Jick et al., 2000; Rockwood et al.; 2002; Wolozin et al., 2000). Thus, it is of interest if statins are able to affect cholesterol metabolism in the brain.

Apparently, the ability of simvastatin to permeate through the bood-brain barrier (BBB) seems to be reasoned by its higher lipophilicity (Tsuji et al., 1993). despite the fact that the active forms of simvastatin and pravastatin have similar chemical structures (Serajuddin et al., 1991). In contrast to pravastatin, applied in its active acid form (sodium-salt), simvastatin is an inactive lactone prodrug that is converted in vivo to the active open-ring acid form.

According to a recent publication both, simvastatin and pravastatin, were found in cerebral cortex after administration of high dosages. Within three weeks, the levels of cholesterol were significantly reduced (Johnson-Anuna et al., 2005).

Cholesterol synthesis can be evaluated by measurement of mRNA expression levels of HMG-CoA- reductase, changes of cholesterol precursors like lathosterol or its ratio to cholesterol (Björkhem et al., 1987). Cholesterol elimination from the brain can be evaluated by measurement of 24(S)-hydroxycholesterol (24S-OH-Chol) (Björkhem et al., 1999). In contrast to cholesterol, 24S-OH-Chol is able to cross the blood-brain-barrier. The gene

responsible for its formation, cholesterol 24(S)-hydroxylase (CYP46A1), may be of key importance for maintenance of cholesterol homeostasis in the brain (Lund et al., 1999).

This study was performed to compare a) the distribution of simvastatin and pravastatin within the different compartments: brain, liver, and serum, and b) the effects on cerebral as well as peripheral cholesterol metabolism after short-term, high-dose treatment. The expression of additional target genes in brain and liver samples involved in cholesterol transport and elimination was determined as well.

Methods

Animals

The animal experiments were approved by the local Animal Ethics Committee (Karolinska University Hospital, Huddinge, Sweden) in accordance with recommendations of the Federation of European Animal Science Association and European legislation. Male mice of the inbred strain C57/BL6 had free access to standard rodent diet and water. The animals were alternately maintained in darkness from 6.00 p.m. to 6.00 a.m.. Twenty-four mice (three months of age) were randomized into four groups of six animals each. Each mouse received 250 µl of aqueous pravastatin-sodium solution (200 mg/kg body weight), simvastatin (100 mg/kg body weight), water or polysorbate 80 (10%, g/g in water) by oral gavage twice a day. Simvastatin was purchased from Merck KG (Darmstadt, Germany). Pravastatin was generously donated by Sankyo Co, Ltd, (Tokyo, Japan). Simvastatin was dissolved in polysorbate 80 (10% g/g in water). The dosage of statins used in the present investigation was chosen according to performance in a set of preliminary experiments with three different dosages of pravastatin-sodium and simvastatin applied to mice of the same strain, sex and age. Lower doses of simvastatin or pravastatin had no effect on the levels of lathosterol or expression levels of HMG-CoAR in the brains of those animals (data not shown). The treatment period lasted three days. The weight of the animals remained constant within this time. About six hours after last administration of the drug or the vehicle the animals were sacrificed via neck break after anesthesia with carbon dioxide gas. Blood, liver, and brains were collected immediately. Livers were minced and flash frozen. The brains were separated into hemispheres for further analysis. Tissue samples were kept frozen at -70°C until analysis. Blood was centrifuged to obtain serum and kept frozen at -20°C.

Analysis of pravastatin and simvastatin in brain, liver, and serum by LC/MS

Pravastatin acid (LKT laboratories, St. Paul, MN, USA), its positional isomer biotransformation product pravastatin-isoacid, pravastatin lactone (LKT laboratories, St. Paul, MN, USA), simvastatin acid (ß-hydroxy simvastatin) (Mikromol, Luckenwalde, Germany), and simvastatin lactone (USP, Rockville, MD, USA) were quantified via HPLC tandem MS. A previously described method was used to determine simvastatin and simvastatin acid (Zhao et al., 2000). The method was modified to determine pravastatin and its lactone form. The statins were extracted by liquid-liquid cartridge extraction with a Chem- Elut™ column (Varian , Zug, Switzerland). Separation and quantification was conducted using a TSQ® 7000 (Finnigan, San Jose, CA, USA) equipped with an Uptisphere® C_{18} column (125 x 2 mm, 5 µm; Interchim, Montlucon, France). A mixture of 1 mM ammonium acetate buffer (pH 4.5) and acetonitrile was used as mobile phase for determination of pravastatin, pravastatin-isoacid, and pravastatin lactone. The run time was set for 12.0 minutes with a flow rate of 250 µl/min. At the beginning the eluent consisted of ammonium acetate buffer (pH 4.5) and acetonitrile (70/30, v/v). After five minutes, the eluent was changed to ammonium acetate buffer (pH 4.5) and acetonitrile (60/40, v/v). After nine minutes, the gradient was changed to 50/50 (v/v) for 50 seconds and finally set back to 70/30 (v/v) until the end of the run. Elution of simvastatin and simvastatin acid was performed in an isocratic mode using the same eluent of ammonium acetate buffer (pH 4.5) and acetonitrile (33/67; v/v) with a run time of 12.5 minutes and a flow rate of 200 µl/min. Following electrospray ionization of analytes, the consecutive product ions were measured using selective reaction monitoring. The mass spectrometer was operated in a negative ion mode for quantification of simvastatin acid (ß-hydroxy simvastatin; m/z 435.3-> 319) and pravastatin (m/z 423.3-> 321). Positive ion mode was performed for determination of simvastatin (m/z 419.3-> 285) and pravastatin lactone (m/z 393.3-> 345). Triamcinolon (Bristol-Myers Squibb, Zurich, Switzerland) (m/z 407.3->287) was used as internal standard for determination of pravastatin according to a previously

described method (Otter and Mignat, 1998). Lovastatin acid (Mikromol, Luckenwalde, Germany) (m/z 421.3-> 319) and lovastatin (USP, Rockville, MD, USA) (m/z 405.3-> 285) were used as internal standards for quantification of simvastatin acid and simvastatin, respectively. The concentrations of statins (acids and lactones), except for pravastatin-isoacid, were calculated due to single point addition of standards for brain and liver analysis. Pravastatin-isoacid was calculated using a calibration curve. The calibration range for determination of statins (lactone and acid form) in mice serum was 0-5 µg/mL for pravastatin as well as simvastatin.

Extraction and analysis of sterols in brain, liver, and serum by GC-MS

Cholesterol, its precursor lathosterol, its metabolite 24S-OH-Chol, and the plant sterols campesterol and sitosterol were extracted from brain, liver, and serum by chloroform/methanol and determined after derivatization to the corresponding trimethylsilyl-ethers by gas chromatography- flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) as reported previously (Lütjohann et al., 2004). Dry weight of brain and liver specimen was determined after drying them to constant weight overnight in a Speedvac^{TM} (Servant Instruments, Inc., Farmingdale, NY, USA) ultracentrifuge dryer.

Plant sterols (phytosterols), such as campesterol (24-methyl-cholest-5-ene-3ß-ol) and sitosterol (24-ethyl-cholest-5-ene-3ß-ol), are C-24-alkyl-homologues of cholesterol. They are always of dietary origin and cannot be synthesized within the mammalian body (Igel et al., 2003). They are absorbed by the intestine via ATP-binding cassette (ABC) G5/G8 tandem transporter, carried by the same lipoproteins like cholesterol in the circulation, and are excreted from the hepatocyte into the bile/feces, again by ABC G5/G8 (Lee et al., 2001). Plant sterols can be found in all tissues and fluids of the mammalian body (Lütjohann et al., 2004; Yu et al., 2004). Their presence in the brain requires their passage across the blood-brain barrier. As exogenous substances, they can be used a) to assess the integrity of the blood

brain barrier in respect of sterol passage and b) ascertain that sterol concentrations are indeed of brain origin and not artefacts from brain supplying blood vessels.

Lipoprotein and liver enzyme analysis in serum samples

Serum from each animal was assayed for triglycerides, creatinine kinase, alanine amino transferase, bilirubin and alkaline phosphatase by routine assays in clinical chemistry.

Total RNA preparation and real-time Reverse-Transcriptase (RT) PCR analysis

Total RNA was extracted from frozen liver and brain samples using the Quick Prep™ Total RNA Extraction Kit (Amersham Biosciences, Buckinghamshire, UK). RNA quality was verified by electrophoresis in a 2.2M formaldehyde/MOPS 1.2 % agarose gel. Three micrograms of Dnase-treated total RNA (Rnase-free Dnase Set, RNeasy® Protect Mini Kit ; Qiagen, Hilden, Germany) was reverse-transcribed into cDNA by using SuperScript III Rnase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and Random primers (Promega, Madison,WI, USA) according to the manufacturers recommendations. Relative mRNA amount was quantified through singleplex real-time rt PCR analysis on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using hypoxanthine guanine phosphoribosyl transferase (HPRT) as internal standard.

The ABC transporter A1 (ABCA1) plays a key role in the reverse cholesterol transport by mobilizing cholesterol from the peripheral tissues to liver. It controls the efflux of intracellular cholesterol to apoAI, the major apolipoprotein of HDL (Brewer et al., 2004). Apolipoprotein E (ApoE) is the predominant apolipoprotein in the brain and participates in cholesterol redistribution from cells with excess cholesterol to those requiring cholesterol. (Mahley, 1988).

HPRT, HMG- CoA reductase, HMG- CoA- synthase, ABCA1, CYP46A1, and LDL-R were amplified with gene-specific primers and Taq Man® Universal PCR master mix

(Applied Biosystems). Amplification of HPRT and ApoE was performed with gene specific primers and SYBR Green® Universal PCR Master Mix (Applied Biosystems). For SYBR Green® assays, amplification of a single PCR product was verified by a dissociation protocol obtained after each run. All PCR amplifications were performed in triplicates in accordance with the manufacturer`s instructions. Relative mRNA levels were calculated according to the $\Delta\Delta C_T$ (Comparative C_T) method as described by the manufacturer (Applied Biosystems; User Bulletin # 2: Relative Quantitation of Gene Expression). To compare the results of controls with statin treated animals, the relative mRNA expression ratios of controls were set as 1.0 (Table 1B). A relative expression ratio of 1.0 is equivalent to one-hundred percent.

Statistical analysis

All statistical procedures were performed with the statistical package for the social sciences software SPSS 12.0 (SPSS Inc., Chicago, IL). The non- parametric Mann- Whitney- test was used to analyze differences between the groups due to the small groups of animals and considered significant at a level of $p < 0.05$. Percentage differences between the different parameters were calculated by the differences between the mean values. Correlations between parameters were calculated by Pearson´s correlation coefficient.

Results

The data from both control groups (water and polysorbate) were combined, as no significant differences in the determined parameters (sterols and mRNA´s) between the two control groups could be found. However, one simvastatin treated animal had to be excluded from the statistical evaluations due to extremely high simvastatin concentrations in brain, liver and serum and increased serum alanine amino transferase and bilirubin levels (data not shown). One animal from the pravastatin group and one animal from the simvastatin group had to be excluded from the mRNA expression analyses due to RNA quality impairment.

Concentration and distribution of statins and metabolites in brain, liver, and serum

The efficacy of treatment with statins is commonly proven by monitoring of lipid parameters such as total or LDL-cholesterol. Here, we measured concentrations of statins and their metabolites in brain, liver and serum, in order to examine the amount and distribution of these compounds within the different compartments and to correlate this data with effects on other markers of cholesterol metabolism.

The concentrations of statins (acids and lactones) showed a broad interindividual range within the different tissues and serum (Table 1A). While the statins in serum were exclusively present in their active acid forms, simvastatin and pravastatin appeared in brain and liver as lactones and acids. Serum concentrations of the acid and isoacid form of simvastatin and pravastatin did not differ, despite the fact that a) the orally applied amount of pravastatin was twice as high as simvastatin and b) the concentrations of total pravastatin (lactone + acid + isoacid) in the liver were on average 3.5 fold higher compared with those of simvastatin. Assuming that these drugs reach the brain exclusively from the periphery via the serum, we conclude that simvastatin and pravastatin penetrate the blood-brain barrier to a similar degree in these mice under the conditions presented here.

A high correlation of body weight corrected simvastatin concentrations was found between brain and liver (r = 0.967; $p = 0.007$), serum and brain (r = 0.961; $p = 0.009$), as well as serum and liver (r = 0.937; $p = 0.019$). In contrast, no such correlations were found for pravastatin. This finding is consistent with the different distribution of simvastatin and pravastatin in the various compartments.

Simvastatin affects brain cholesterol synthesis

Short-term treatment with high-dose simvastatin as well as pravastatin did not change the levels of cholesterol in whole brain homogenates (Table 1A). Compared to the control group, simvastatin significantly decreased the levels of the cholesterol precursor lathosterol (-28%, $p = 0.002$) as well as its ratio to cholesterol (–29%, $p = 0.002$), indicating a reduction of cholesterol synthesis in the brain. Compared to controls, inhibition of HMG-CoAR was followed by increased mRNA expression levels of HMG-CoAR (+59%; $p < 0.001$) (Table 1B). Additionally, mRNA expression of the ABCA1 in brains of simvastatin treated mice was higher than in controls (+48%, $p < 0.001$).

Although local concentrations of pravastatin were on average 1.3-fold higher than those found in the brains of simvastatin treated animals, pravastatin treatment did not decrease the levels of the cholesterol precursor lathosterol nor its ratio to cholesterol, and neither did it upregulate levels of HMG-CoAR mRNA expression. Surprisingly, HMG-CoAR mRNA expression levels were even downregulated (-29%, $p < 0.001$ versus controls). The levels of lathosterol in the brains of simvastatin treated mice were slightly but significantly lower than those of pravastatin treated animals (-20%, $p = 0.045$). The mRNA expression levels of HMG-CoA synthase, CYP46A1, and Apo E in the brain were not affected neither by simvastatin nor by pravastatin treatment.

We can conclude that whole brain cholesterol turnover does not seem to be affected by short-term, high-dose statin treatment, as levels of cholesterol and its metabolite, 24S-OH-Chol, remained unchanged (Table 1A).

Determination of plant sterols in the different compartments

Differences in peripheral plant sterol concentrations after short-term statin treatment were not reflected in the brain. Hence, high-dose statin treatment does not seem to influence the passage of plant sterols across the blood-brain barrier or their accumulation within this short time interval. The ratios of campesterol to sitosterol in liver and serum did not differ despite the fact that their absolute concentrations are lower in statin treated animals (Table 1A). However, the ratio of campesterol to sitosterol in the brain was significantly higher than the corresponding serum ratios (6.10 ± 1.65 vs. 3.49 ± 0.26; $p < 0.001$). From the latter, we conclude that either a) the passage of campesterol across the BBB is more favored than it is for sitosterol or b) the release of sitosterol from the brain is higher than for campesterol. In any case, the comparison of campesterol and sitosterol concentrations in brain and serum indicates that possible contamination (artefacts) with blood from measurement of whole brain sterol content is of minor importance.

Effects of simvastatin and pravastatin on peripheral cholesterol metabolism

High-dose statin treatment did not cause a reduction of cholesterol levels in the liver, or in serum. (Table 1A). Levels of triglycerides in serum were also not affected (data not shown). However, in contrast to the brain, simvastatin treatment did not result in any significant changes in lathosterol levels in the liver, while in the serum, the levels of lathosterol were significantly decreased by 33% ($p = 0.002$),. Nevertheless, there was a significant upregulation of HMG-CoA-reductase mRNA expression in the liver (+131%, $p = 0.019$). Apart from this upregulation, HMG-CoA-synthase mRNA expression ratios were also

increased (+54%, p = 0.019). The mRNA expression levels of Apo E (-21%; p < 0.001), ABCA1 (-16%, p = 0.019), as well as of LDL receptor (-39%, p = 0.019) were significantly decreased compared to controls after high-dose, short-term treatment with simvastatin. The concentrations of 24S-OH-Chol and the 24S-OH-Chol to cholesterol ratio in the liver were significantly decreased by 24% (p = 0.045 and p = 0.035, respectively) compared to controls. Mice fed with pravastatin displayed more profound effects on cholesterol synthesis in the liver compared to the effect observed in mice fed with simvastatin. The absolute levels of lathosterol as well as its ratio to cholesterol were significantly decreased by 66% (p = 0.011) and 69% (p = 0.005), respectively. In serum, pravastatin treatment caused a reduction of lathosterol concentrations of 25% (p = 0.044) compared to untreated animals (controls). Liver HMG-CoA-reductase mRNA expression levels (+256%, p < 0.001) and HMG-CoA-synthase mRNA expression levels (+186%, p < 0.001) were significantly increased after pravastatin treatment. The expression of LDL receptor mRNA in the liver was significantly increased under pravastatin compared to controls (+43%; p = 0.007) and to simvastatin (p = 0.045). The ABCA1 mRNA expression levels increased due to pravastatin treatment (+58%, p < 0.001). The absolute levels of 24S-OH-Chol remained unchanged in the liver, but its ratio to cholesterol was significantly higher compared to simvastatin treated animals (p = 0.028). Correlation analysis revealed a significant correlation between LDL receptor and HMG-CoAR expression in the liver of the pravastatin group (r = 0.821; p = 0.045). Control animals showed a significant correlation between mRNA levels of HMG-CoAR and synthase (r = 0.739; p = 0.009) as well as between HMG-CoA synthase and LDL receptor (r = 0.781; p = 0.005). In the control group, there was a high correlation between liver and brain ApoE mRNA (r = 0.878; p < 0.001).

Discussion

Short-term, high-dose oral administration of simvastatin clearly affects brain cholesterol synthesis in male C57/BL6 mice as evaluated by decreased levels of the cholesterol precursor lathosterol and increased mRNA expression ratios of HMG-CoAR in whole brain homogenates. In contrast, pravastatin failed to show an inhibitory effect on cholesterol synthesis in the brain.

There are several studies indicating that simvastatin but not pravastatin is able to penetrate the blood-brain barrier (Saheki et al., 1994; de Vries and Cohen, 1993) and causing CNS side effects such as sleep disturbances. The ability to cross the BBB is thought to be caused by the difference in lipophilicity.

The active forms of simvastatin and pravastatin have similar chemical structures, but pravastatin is approximately 100 times less lipophilic than simvastatin (Serajuddin et al., 1991). In theory pravastatin should therefore be regarded as being unable to cross the blood-brain barrier to a significant extent (Guillot et al., 1993). Pravastatin is metabolized mainly via hydroxylation and its metabolites are even more hydrophilic, whereas the main metabolite of simvastatin is the still lipophilic active form, simvastatin acid.

Here, we show that pravastatin and its corresponding lactone are detectable in the brain in concentrations resulting in effects on cholesterol synthesis for simvastatin. In contrast to simvastatin, and despite of the relatively high levels of the drug measured in the brain, pravastatin treatment had no significant effect on the cholesterol precursor lathosterol in the brain. Interestingly, the HMG-CoAR mRNA levels were slightly decreased instead of being increased by pravastatin. The reason for the different responses might be the varying transportation of the two statins across blood-tissue barriers and accumulation in cell compartments (Tsuji et al., 1993). It was proposed that pravastatin is unable to penetrate cell membranes but instead accumulates inside the membrane in tissue other than the liver (Koga et al., 1990). In contrast to simvastatin, it seems likely that the high-dose pravastatin treatment

caused a toxic effect in our study resulting in the suppression of HMG-CoAR mRNA expression. However, the underlying reason for this is unclear and needs to be further elucidated. We can only speculate that pravastatin is unable to affect cholesterol synthesis in the brain since it might not reach the target locus.

We treated the animals with dosages 75 and 300 times higher (for simvastatin and pravastatin, respectively) than therapeutical doses in humans per day. According to some pilot studies performed in our laboratory, lower doses of simvastatin applied to animals of the same strain and age did not affect brain cholesterol synthesis. Consequently, we are quite certain that we reached drug equilibration of simvastatin between blood and brain. However, it is not clear whether this was also the case for pravastatin. Nevertheless, we found comparable concentrations of simvastatin and pravastatin in serum and whole brain homogenates.

In the present experiment the cholesterol levels in the brain were not altered by treatment with pravastatin or simvastatin. It is known that brain cholesterol has a remarkably long half life (Andersson et al., 1990). Thus, treating mice with high doses of statins for three days may not be sufficiently long enough to change the total cholesterol levels in this organ. In previous animal studies a longer duration time of treatment resulted in no change of whole brain cholesterol content (Petanceska et al., 2002; Lütjohann et al., 2004). One does not necessarily expect any change in the concentration of cholesterol with these manipulations in the CNS. Reduced levels of cholesterol in the brain cortex were described after treatment with high-dose simvastatin and pravastatin. However, the expression of HMG-CoAR mRNA in the cortex was not different from controls (Johnson-Anuna et al., 2005). The vast majority of cholesterol is in myelin while less than 2% of the cholesterol is in the neuron pool that is of interest (Dietschy and Turley, 2004). Assuming a local effect of statins, we have to consider that altering cholesterol levels in the brain might have hazardous consequences regarding the important role of cholesterol in neuronal growth and signal transduction. In those studies

which show a gross change in cholesterol content in the brain, a serious toxic effect, resulting in demyelination, could have occured.

In this study, significant effects on the cholesterol precursor lathosterol in the brain were observed as a result of simvastatin treatment. It is noteworthy, that in cells and organs, concentrations of cholesterol precursors and cholesterol itself differ with a factor of 1000. However, the changes in precursor levels as presented in this study reflect the potency of simvastatin to alter brain cholesterol synthesis. The pool of cholesterol in the whole brain appears to remain stable and brain cholesterol homeostasis seems to be well regulated, ensuring the demand for cholesterol supply in this organ.

The inhibitory effects on cholesterol synthesis in the brain due to simvastatin treatment were accompanied by a compensatory increase in HMG-CoAR mRNA expression as well as an increased level of ABCA1 mRNA. Levels of ApoE mRNA were not affected.

In a previous study, high-dose treatment of transgenic PSAPP mice with atorvastatin decreased ApoE levels in the brain (Petanceska et al., 2003). However, in the present study the ApoE mRNA levels were, not affected by either of the two statins. This might be due to the short duration of treatment in our study, the different mouse strain, the genetic background, or the statins used in these experiments. However, the expression of mRNA is not necessarily correlated to protein abundance levels or enzyme activity (Gygi et al., 1999). Neither expression ratios of CYP46A1 mRNA, nor levels of 24S-OH-Chol were significantly affected by the treatment, indicating that the inhibition of cholesterol synthesis had no effect on the most important mechanism for elimination of cholesterol from the brain. In a previous study in which guinea pigs were treated with the two statins, a slight but significant effect on brain levels of 24S-OH-Chol was observed (Lütjohann et al., 2004). This discrepancy between the guinea pig experiment and our study may possibly be due to the different treatment periods. In mice, however, the metabolism of this oxysterol is different. While in mice, only about 50 % of circulating 24S-OH-Chol originate from the brain (Meaney et al.,

2000), in humans 24S-OH-Chol is exclusively of brain origin (Björkhem, 1999). Thus, comparing results of studies on cholesterol metabolism from mice, guinea pigs and humans has limitations. For instance, in wild-type mice, plasma levels of cholesterol are relatively low and cholesterol is predominantly found in the HDL fraction (Terpstra et al., 1982) whereas in humans, most cholesterol is located in the LDL fraction (Fielding and Fielding, 1982).

Further differences between human and murine model are supported by our finding that there was no change of absolute cholesterol levels in serum and liver due to high-dose statin treatment which is in accordance with previous reports (Dietschy and Turley, 2002; Endo et al., 1979; Kita et al., 1980). Nevertheless, other studies report slight reductions in serum/plasma cholesterol levels in mice under statin treatment (Park et al., 2003; Petanceska et al., 2002). This might be explained by the short-term treatment performed in our study.

In the liver, the levels of pravastatin were found to be markedly higher than levels of simvastatin. This finding can not solely be attributed to the doubling of the dose. The high levels of pravastatin in the liver may be related to the fact that this specific statin is subjected to an extensive carrier-mediated uptake in hepatocytes (Niemi et al., 2004). This is in contrast to most other statins which apparently enter cells mainly through passive diffusion. The mRNA expression ratios of HMG-CoAR and HMG-CoA synthase levels were significantly increased by both statins, with more marked effects for pravastatin than for simvastatin. In the pravastatin treated animals also ABCA1 and LDL receptor mRNA levels were increased. Likewise, the levels of lathosterol were reduced to a higher degree by pravastatin in the liver. A stronger decrease of lathosterol concentrations in serum by simvastatin might indicate a more pronounced inhibition of cholesterol synthesis in the periphery apart from the liver. Total simvastatin concentrations in brain, liver and serum correlated significantly whereas there was no such effect seen in pravastatin treated animals. The different effects of simvastatin and pravastatin on cerebral and peripheral cholesterol synthesis may in part be

due to the differences in metabolism and lipophilicity, as well as the different uptake and tissue selectivity (van Vliet et al., 1995; Ziegler and Hummelsiep, 1993).

We have shown conclusively that high doses of simvastatin affect brain cholesterol synthesis in mice, and that this effect is most likely the result of a direct passage of the drug across the blood-brain barrier and a local inhibition of cholesterol synthesis in the brain. A similar concentration of pravastatin failed to show an inhibitory effect on brain cholesterol synthesis. However, the amount of whole brain cholesterol was not affected under the applied conditions. The potency of the different statins to inhibit cholesterol synthesis in the CNS seems to depend on their lipophilicity and distribution in the brain. The possibility must be considered that administration of lipophilic statins like simvastatin in high doses may alter brain cholesterol synthesis also in humans.

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Footnotes

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Table 1A:
Concentrations of statins and metabolites [per g wet weight tissue or mL serum] and levels of sterols [per mg dry weight tissue or volume serum] and the ratios of sterols to cholesterol (R_sterol) in vehicle-fed (Control), Simvastatin and Pravastatin treated animals

		Brain				Liver				Serum		
		Control (n=12)	Simvastatin (n=5)	Pravastatin (n=6)		Control (n=12)	Simvastatin (n=5)	Pravastatin (n=6)		Control (n=12)	Simvastatin (n=5)	Pravastatin (n=6)
Compound:												
Lactone	[pmol/g]	n.d.	14.7 ± 12.5	6.36 ± 8.93	[pmol/g]	n.d.	17.9 ± 13.6	98.2 ± 56.5	[pmol/mL]	n.d.	n.d.	n.d.
Acid	[pmol/g]	n.d.	50.1 ± 59.6	52.0 ± 60.0	[pmol/g]	n.d.	552 ± 531	316 ± 231	[pmol/mL]	n.d.	221 ± 121	81.8 ± 61.3
Isoacid	[pmol/g]	n.d.	n.d.	27.8 ± 31.9	[pmol/g]	n.d.	n.d.	1600 ± 513	[pmol/mL]	n.d.	n.d.	107 ± 88
Total	[pmol/g]	n.d.	64.8 ± 69.3	86.2 ± 71.4	[pmol/g]	n.d.	570 ± 543	2014 ± 701	[pmol/mL]	n.d.	221 ± 121	190 ± 88
Sterols and R_sterol:												
Cholesterol	[µg/mg]	54.3 ± 3.1	55.1 ± 4.0	53.1 ± 2.1	[µg/mg]	7.5 ± 1.0	7.5 ± 1.3	7.4 ± 0.6	[mg/dL]	119 ± 12	103 ± 24	107 ± 22
Lathosterol	[ng/mg]	89.4 ± 6.3	64.5 ± 11.3[b]	80.5 ± 12.7[d]	[ng/mg]	9.9 ± 5.4	5.6 ± 3.6	3.4 ± 1.8[b]	[µg/dL]	69 ± 16	46 ± 3[b]	51 ± 13[a]
R_Lathosterol	[µg/mg]	1.65 ± 0.12	1.18 ± 0.21[b]	1.52 ± 0.24	[µg/mg]	0.90 ± 0.45	0.52 ± 0.38	0.29 ± 0.12[a]	[µg/mg]	0.58 ± 0.12	0.48± 0.08	0.47 ± 0.13
24(S)-OH-Chol	[ng/mg]	193 ± 39	185 ± 30	194 ± 71	[pg/mg]	130 ± 31	99 ± 20[a]	132 ± 27	[ng/mL]	38 ± 4	37 ± 3	38 ± 3
R_24(S)-OH-Chol	[ng/mg]	3.56 ± 0.69	3.37 ± 0.58	3.66 ± 1.41	[ng/mg]	17 ± 4	13 ± 2[a]	18 ± 4[d]	[ng/mg]	32 ± 3	37 ± 7	37 ± 8
Campesterol	[ng/mg]	58.2 ± 9.7	57.7 ± 11.8	59.5 ± 4.7	[ng/mg]	247 ± 40	186 ± 47[a]	199 ± 13[b]	[mg/dL]	3.6 ± 0.76	2.4 ± 0.78[b]	2.7 ± 0.70[b]
Sitosterol	[ng/mg]	9.4 ± 1.5	12.9 ± 5.3	9.3 ± 2.3	[ng/mg]	52.8 ± 8.0	42.8 ± 10.6	42.4 ± 4.7[c]	[mg/dL]	1.0 ± 0.20	0.71 ± 0.29[b]	0.78 ± 0.23[b]

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ compared to controls
[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ compared to Simvastatin
n.d. = not detectable
Data are expressed as mean ± SD.

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Table 1B:
Relative expression ratios of mRNA´s in the brain and liver after treatment with simvastatin and pravastatin compared to controls (n=12)

	Brain			Liver		
	Control (n=12)	Simvastatin (n=5)	Pravastatin (n=6)	Control (n=12)	Simvastatin (n=5)	Pravastatin (n=6)
ıRNA expression Ratios:						
lMG CoA reductase	1.0	1.59 ± 0.21[c]	0.71 ± 0.24 [c, d]	1.0	2.31 ± 1.60[a]	3.56 ± 1.64[c]
lMG CoA synthase	1.0	1.35 ± 0.55	0.99 ± 0.37	1.0	1.54 ± 0.68[a]	2.86 ±1.67[c]
ıpoE	1.0	1.01 ± 0.51	0.92 ± 0.22	1.0	0.79 ± 0.06[c]	1.05 ± 0.27[d]
ıBCA1	1.0	1.48 ± 0.32[c]	0.80 ± 0.53	1.0	0.84 ± 0.46[a]	1.58 ± 0.44[c, d]
:DL-R	1.0	n.d.[g]	n.d.	1.0	0.61 ± 0.54[a]	1.43 ± 0.44[b, d]
:YP46A1	1.0	0.97 ± 0.34	1.00 ± 0.56	1.0	n.d.	n.d.

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ compared to Controls
[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.001$ compared to Simvastatin
[g] n.d. = not determined
Data are expressed as mean ± SD.