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Cholesterol synthesis in central nervous system of rat is affected by simvastatin as well as by atorvastatin

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Objective: There is evidence to suppose that cholesterol-lowering drugs such as statins might confer protection against dementia, probably via modulation of cholesterol synthesis in the brain. The aim of the present study was to investigate possible influence of two lipophilic statins (simvastatin and atorvastatin) on cholesterol synthesis in selected parts of rat central nervous system (CNS). *Methods:* Three groups of rats were orally treated with simvastatin (10 mg/kg b.wt.), atorvastatin (10 mg/kg b.wt.) or vehicle (aqua) for 9 days. At the end of experiment, brains (for basal ganglia, frontal cortex and hippocampus) and spinal cord were isolated and cholesterol synthesis was determined using the incorporation of deuterium from deuterated water. ANOVA with Fisher's LSD Multiple-Comparison Test and Kruskal-Wallis test were applied for statistical evaluation. $P < 0.05$ was considered statistically significant. *Results:* Significant reductions of cholesterol synthesis rate were detected in both experimental groups (vs. controls) in all studied localisations. Both drugs elicited comparable effects on cholesterol synthesis rate irrespective of the examined tissue. *Conclusions:* This study brings additional evidence of the role of statins in the CNS cholesterol synthesis. The finding that both statins were able to lower brain cholesterol synthesis without altering plasma cholesterol supports the idea of their local action in the brain. For comparison of the effects of statins in the spinal cord and selected parts of brain, the deuterium technique was utilised for the first time.

1. 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. Current therapeutic strategies for AD focus on cognitive deficit alleviation via direct acetylcholinesterase inhibition. Some, but not all (Rea et al. 2005; Zandi et al. 2005) of recent epidemiological reports indicate, that cholesterol-lowering drugs such as statins might confer protection against dementia (Rockwood et al. 2002; Zamrini et al. 2004). Thus, it is of interest whether their action is mediated through a possible effect on brain cholesterol synthesis. In this view, more lipophilic statins that easily cross the blood-brain barrier (BBB) should have higher effectiveness than less lipophilic ones. On the other hand, recent epidemiologic data suggest that there is decreased prevalence of dementia among individuals using statins irrespective of their chemical structure (Wolozin et al. 2000).
This study was performed to test the influence of two statins with different level of lipophilicity (simvastatin and atorvastatin) on cholesterol synthesis rate in various parts of rat brain.

2. Investigations and results

We investigated a possible impact of two statins on cholesterol levels in plasma and cholesterol synthesis in brain (basal ganglia, frontal lobe and hippocampus). Treatment neither with simvastatin nor with atorvastatin produced any decrease of plasma cholesterol ($p = 0.31$, for details see Table 1). Brain cholesterol synthesis in the control group was compared with that in the atorvastatin and simvastatin groups. Treatment with simvastatin as well as with atorvastatin for the dose and time investigated, significantly decreased cholesterol synthesis rates in all CNS parts investigated (for details see Table 2). However, both drugs elicited comparable effects on cholesterol synthesis irrespective of the examined tissue.

Table 1: Cholesterol (mmol/l) in plasma

	Plasma
Control group	1.235; 0.98–2.50
Atorvastatin group	1.690; 1.20–2.35
Simvastatin group	1.625; 1.05–1.93

Results are expressed as median; minimum – maximum

Table 2: FSR (fraction synthesis rate) of cholesterol in various parts of brain

	Hippocampus	Basal ganglia	Frontal lobe	Spinal cord
Control group	0.048 ± 0.0106	0.031 ± 0.0075	0.056 ± 0.0087	0.031 ± 0.0087
Atorvastatin group	$0.028 \pm 0.0066^{***}$	$0.020 \pm 0.0034^{**}$	$0.034 \pm 0.0059^{***}$	$0.017 \pm 0.0053^{***}$
Simvastatin group	$0.035 \pm 0.0076^{***}$	$0.020 \pm 0.0061^{**}$	$0.031 \pm 0.0073^{***}$	$0.017 \pm 0.0045^{***}$

3. Discussion

3.1. Biomarkers of brain cholesterol synthesis

Nearly all CNS cholesterol originates from *in situ* synthesis. As biomarkers of cholesterol homeostasis, total cholesterol, 24S-hydroxycholesterol, lathosterol and 27-hydroxycholesterol are employed. Cholesterol 24-hydroxylase (CYP46a1) in brain is capable of converting cholesterol to 24S-hydroxycholesterol (cerebrosterol), a substance that crosses the BBB and enters the plasma where it can be measured as a marker for cholesterol elimination (Dietschy and Turley 2001). Lathosterol is a cholesterol precursor and its ratio to cholesterol is calculated as an indicator of endogenous cholesterol synthesis (Dietschy and Turley 2001).
However, to estimate the absolute rate of cholesterol synthesis *in vivo*, quantitation of the incorporation rates of either ^2H or ^3H atom (from [^2H] water of [^3H] water) into cholesterol molecule is recommended (Dietschy and Turley 2001). Because ^2H_2O is safe, non-radioactive and can be applied *per os*, it was utilized in the present study.

3.2. Statins and their 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 and pravastatin (−0.22) is regarded as a hydrophilic compound (in brackets: logarithm of the partition coefficient, Corsini et al. 1999; Schachter 2004). Some experimental trials have investigated the effects of different statins on biomarkers of cholesterol synthesis and elimination (their results are summarized in Table 3). Generally speaking, statins have the ability to suppress cholesterol synthesis in the brain (i.e. lower lathosterol levels) without affecting total brain cholesterol. In this aspect it would be interesting to see if the effect 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 favour of the former) on brain cholesterol synthesis (Thelen et al. 2006). Moreover, Johnson-Anuna et al. (2005) demonstrated that although cholesterol levels fail to differ significantly among 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. On the other hand, Lutjohann et al. (2004) suggested that brain cholesterol synthesis in guinea pigs is influenced by simvastatin as well as by pravastatin. We examined the effects of simvastatin and atorvastatin using ten-times lower doses and a somewhat longer duration of treatment (9 days vs. 3 days) than Thelen et al.

Table 3: Experimental studies studying the effect of statins on cholesterol metabolism

	Dosage	Duration of the study	Type of animal	Levels in plasma	Levels in brain
Eckert 2001	Lovastatin 100 mg/kg/day	23 days	Normal mice	∅ cholesterol	↓ cholesterol
			ApoE deficient mice	∅ cholesterol	∅ cholesterol
Pentaceska 2001	Atorvastatin 30 mg/kg/day	8 weeks	Transgenic PSAPP mice	↓ cholesterol	∅ cholesterol (in cortex)
Johnson-Anuna 2005	Lovastatin 100 mg/kg/day	21 days	Normal mice		∅ cholesterol
	Pravastatin 100 mg/kg/day				↓ cholesterol
	Simvastatin 50 mg/kg/day				↓ cholesterol
Lutjohann 2004	Pravastatin 300 mg/day	3 weeks	Guinea pigs	↓ cholesterol ↓ lathosterol/ cholesterol	∅ cholesterol ↓ lathosterol ↓ 24S-OH-chol ↓ lathosterol/cholesterol
	Simvastatin 150 mg/day			↓ cholesterol ↓ lathosterol/ cholesterol	∅ cholesterol ↓ lathosterol ∅ 24S-OH-chol ↓ lathosterol/cholesterol
Thelen 2006	Pravastatin 200 mg/kg/day	3 days	Normal mice	∅ cholesterol ↓ lathosterol ∅ 24S-OH-chol	∅ cholesterol ∅ lathosterol ∅ 24S-OH-chol
	Simvastatin 100 mg/kg/day			∅ cholesterol ↓ lathosterol ∅ 24S-OH-chol	∅ cholesterol ↓ lathosterol ∅ 24S-OH-chol
Franke 2007	Simvastatin 50 mg/kg/day	21 days	Guinea pigs	↓ cholesterol	∅ cholesterol

Legend: ∅ = no change

Results are expressed as mean ± standard deviation, symbols ** and *** denote $p < 0.01$ and $p < 0.001$, respectively (vs. controls).

(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 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 though BBB, whereas those having 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 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 though 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. (2002) 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. 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 measurement of cholesterol flux and by the rates of cholesterol synthesis being too low to be detected by current methods (Dietschy and Turley 2001). Evidently, the relationship between cholesterol homeostasis within and outside the CNS is not elucidated yet.

In the present experiment we have proved, that both statins are able to lower brain cholesterol synthesis without altering plasma cholesterol, 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.

3.3. Cholesterol synthesis in various parts of the CNS

The above mentioned studies have not investigated cholesterol and/or its synthesis in different parts of animal brain. Some of them have examined cerebral cortex (Johnson-Anua et al. 2005; Petanceska et al. 2001) the others whole brain homogenates (Lutjohann et al. 2004; Thelen et al. 2006). We have taken samples from three distinct parts of the rat brain (basal ganglia, hippocampus and frontal cortex) and the spinal cord and found out that cholesterol synthesis rate was the lowest in spinal cord both in the simvastatin and the atorvastatin group.

As to our knowledge, this is the first experimental study comparing the influence of statins on different parts of rat brain using the incorporation of deuterium from deuterated water. In comparison with prior studies we have used much lower statin doses which resemble those administered in humans. For better understanding of the influence of statins on the brain and their involvement in AD pathology, parallel determination of amyloid beta and acetylcholinesterase activity is required in future studies.

4. Experimental

4.1. Animals

Adult male rats of Wistar strain (240g at delivery) were obtained from Biotest, Konárovice, Czech Republic. Animals were housed in the animal quarters for at least 7 days prior to experiments at 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, Velaz, Prague, Czech Republic) except for 16–18 h before and 1 h after experiment, when they were fasted. 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 10% with ^2H_2O (Diraison et al. 1996). Drugs were administered via a metallic gastric probe every day between 9 and 11 a.m for 9 days. For individual dose adjustment, animals were weighed before each application (the average weight of animals over the course of the experiment was 313 g). 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).

4.2. Experimental protocol

The animals were randomly divided into 3 groups, 8 subjects in each. The first (control) group received vehicle only (aqua), the second was administered atorvastatin (10 mg/kg b.wt., Pfizer, Goedecke AG Freiburg, Germany), the third ingested simvastatin (10 mg/kg b.wt., IVAX Pharmaceuticals, Czech Republic). The dosage was adjusted according to previous experiments. The last day of the experiment, 1 h after drug application, the animals were put under pentobarbital intraperitoneal anaesthesia (0.5 mg/g) and were sacrificed by exsanguination (blood withdrawal) from abdominal aorta without delay. Their brains were immediately exteriorised and kept frozen at −20 °C until analysis. Before analysis, samples of the following central nervous system (CNS) parts were isolated: basal ganglia, frontal lobe, hippocampus and spinal cord.

4.3. Clinical chemistry

Individual parts of brain were homogenised using an IKA T10 basic, Ultra-Turrax homogenizer (IKA-Werke, Germany) and extracted according the method of Bligh and Dyer (1959). Briefly, tissue samples were mixed with methanol : water solution (2 : 0.8) and extracted to chloroform using a Stuart rotator (Barloworld Scientific, Stone, UK). The chloroform layer was separated, evaporated to dryness and cholesterol was derivatised using acetylchloride solution in chloroform (1 : 5) for one hour (Liebisch et al. 2006). The mixture was evaporated under nitrogen and the residue containing cholesterol acetate was dissolved in n-hexane for analysis.

Analysis was performed on GC-MS system (Perkin-Elmer, Norwalk, USA) operating in the electron ionisation mode. The injector temperature was set to 300 °C, split ratio 1 : 10, oven 320 °C isothermally, ionisation 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 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, $m_i/m_0 + m_{+1} + m_{+2}$). Then, the average number of deuterium atoms incorporated was calculated $IE_{obs} = m_{+1} + 2m_{+2}$ and compared with maximum number of deuterium atoms (twenty seven) that can be incorporated into cholesterol molecule at given enrichment of plasma water (p).

$$IE_{exp} = 27 \times p$$

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 fractional synthesis rate ($FSR = IE_{obs}/IE_{exp}$) representing a fraction of cholesterol content in tissue, which was synthesized during tracer application period (8 days).
This application period is suitable for tissues with 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.

4.4. Statistical analyses

The data were processed by the programs NCSS 2004 and Statistica. After testing the normality and homogenicity of data, Analyses Of Variance with Fisher's LSD Multiple-Comparison Test and Kruskal-Wallis Test were applied for statistical evaluation. The chosen level of significance was $\alpha = 0.05$.

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