Short Communication

Title

Alendronate lowers cholesterol synthesis in the central nervous system of rats – a preliminary study.

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Running title: Alendronate lowers brain cholesterol synthesis

Summary

Introduction: Nitrogen-containing bisphosphonates were found to inhibit farnesyl diphosphate synthase - an essential enzyme in the cholesterol biosynthesis pathway, but their effect on cholesterol synthesis *per se* in the central nervous system (CNS) remains unknown. The aim of the present study was to examine possible influence of a representative agent alendronate on cholesterol synthesis rates in selected parts of rat CNS and blood cholesterol level.

Methods: 2 groups of rats were orally administered either alendronate (3 mg/kg b.wt.) or vehicle (aqua) for 9 days. At the end of experiment, brain (for basal ganglia, frontal cortex and hippocampus) and spinal cord were isolated and cholesterol synthesis was determined using the technique of deuterium incorporation from deuterated water. For statistical evaluation, ANOVA with Fisher's LSD Multiple-Comparison Test and Kruskal-Wallis Test were applied.

Results: In the alendronate group significant reductions of cholesterol synthesis rates were detected in frontal cortex, hippocampus and spinal cord ($p<0.001$). However, the experimental treatment did not produce a significant alteration in the levels of plasma cholesterol.

Conclusions: This study brings the first experimental evidence of the inhibition of CNS cholesterol biosynthesis with alendronate.

Key words

Brain cholesterol synthesis, Bisphosphonates, Alendronate, Deuterium oxide

Cholesterol has been widely discussed as a molecule participating in the pathophysiology of neurodegenerative diseases. The putative role of cholesterol in Alzheimer´s disease (AD) is supported by reports indicating a decreased risk of this condition by cholesterol-lowering drugs – statins (Rockwood *et al.* 2002, Zandi *et al*. 2005). Statins block a rate-limiting step in the cholesterol biosynthesis cascade via 3-hydroxymethyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibition. As a consequence, the production of amyloid-β (the characteristic AD protein) may be diminished (Fassbender *et al.* 2002, Simons *et al*. 1998).

The latest and most potent bisphosphonates, nitrogen-containing bisphosphonates (N-BPs, e.g. alendronate) were found to be potent inhibitors of cholesterol biosynthesis from mevalonate (Amin *et al.* 1992). In the corresponding pathway, the enzyme farnesyl diphosphate synthase (= isopentenyl transpherase) has recently been indentified as the molecular target of N-BPs (Rezka and Rodan 2004). Although the use of bisphosphonates has been indicated primarily and principally for treatment and prevention of bone health disturbances such as osteoporosis, the similarity with statins regarding the mechanism of action suggests an analogy in the alternative use of N-BPs.

We hypothesised that alendronate lowers cholesterol biosynthesis in the central nervous system (CNS) in a similar way to statins. Hence, the aim of the present study was to determine possible modifications in blood cholesterol levels and cholesterol synthesis rates within selected parts of rat brain and spinal chord after exposure to alendronate.

We have used adult male rats of Wistar strain (240g at delivery). The rats had free access to standard laboratory rat chow pellets except for 16–18h before and 1h after experiment, when they were fasted. The second day rats received a loading dose of deuterated water (35mL/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 9:00 and 11:00 a.m. for nine days. For individual dose adjustment, animals were weighed before each application. 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. Animals were randomly divided into two groups, eight subjects in each. The first (sham) group received vehicle only (aqua), whereas the second was administered alendronate (3 mg/kg b.wt., MSD, Merck Sharp & Dohme B.V., Netherlands). The last (ninth) day of experiment, 1h after drug application, animals were put under pentobarbital intraperitoneal anaesthesia (0.5mg/g) and were sacrificed by exsanguination (blood withdrawal) from abdominal aorta without delay. Their brain and spinal chord were immediately exteriorised and basal ganglia, frontal lobe and hippocampus isolated. Individual parts of brain were homogenised using KIA T10 basic, Ultra-Turrax homogenizer (IKA-werke, Germany) and extracted according the method of Bilgh and Dyer (Bligh and Dyer 1959). Briefly, tissue samples were mixed with methanol: water solution (2:0.8) and extracted to chloroform using 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 residue containing cholesterol acetate was dissolved in n-hexane for analysis. Analysis was performed on GC-MS system (Perkin-Elmer, Norwalk, USA) operating in electron ionisation mode. The injector temperature was set to 300ºC, slit ratio 1:10, oven 320ºC isothermally, ionisation source 280ºC. 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). 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. For statistical evaluation, descriptive measures, normality tests followed by ANOVA with Fisher's LSD *post hoc* Multiple-Comparison Test (brain data) and

Mann-Whitney test (plasma data), were applied. The employed programs were NCSS 2004, Statistica and GraphPad InStat.

Treatment with given dose of alendronate for nine 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). However, the administered dosage significantly ($p<0.001$) decreased the rate of cholesterol synthesis in three distinct parts of the CNS (hippocampus, frontal lobe and spinal cord, for details see Table 1).

The specified areas of brain were selected respecting their relevance in AD pathophysiology - hippocampus, basal ganglia and frontal lobe are the most severely affected structures by degeneration of cholinergic system.

Very limited data are available concerning the potency of bisphosphonates to inhibit cholesterol biosynthesis. In humans, Canigga *et al.* were the first to demonstrate the ability of supratherapeutic doses of etidronate to lower serum cholesterol and total lipid levels (Canigga *et al*. 1974). These findings were supported in a study by Montagnani *et al.*, who documented an induction of a weak decrease in total cholesterol and cholesterol-shift from the low density lipoprotein to high density lipoprotein fraction in patients with Paget´s bone disease by nine-month treatment with pamidronate three times 60 mg i.v. (Montagnani *et al.* 2003). In (ovariectomized) rats, on the other hand, three week-administration of alendronate (3 mg kg^{-1} p.o. daily) did not elicit significant effect on blood cholesterol levels (Frolik *et al*. 1996). The results of the present study are in keeping with the latter findings and indicate that the dosage used (3 mg kg^{-1} p.o. daily for nine days) is either insufficient to produce significant effects on blood cholesterol levels or challenge the ability of alendronate to lower plasma cholesterol in rats.

With regard to CNS, the finding of lowered cholesterol biosynthesis due to alendronate treatment is, to our best knowledge, unique. In this respect alendronate (a BBB penetrating drug) mimics the action of lipophilic statins (as demonstrated on by the decrease of lathosterol - Lutjohann *et al.* 2004, Simons *et al.* 2005, Hoglund *et al.* 2005) and analogically suggests a possible connection with cholinergic neurotransmission. Firstly, the levels of total brain cholesterol were shown to positively correlate with the amount of amyloid beta (Aβ) (Refolo *et al.* 2000), a peptide known for its ability to increase the activity of acetylcholinesterase (AChE) *in vitro* (Hu *et al.* 2003). Secondly, alendronate also suppresses AChE activity in frontal cortex (the site of the highest Aβ accumulation) as has recently been demonstrated in our previous study (Cibičková *et al.* 2007). And finally, build-up of Aβ peptide is associated with a reduction of cholinergic transmission, which is characteristic for AD. Since other cholesterol-lowering drugs (statins) play a putative preventive role in AD, these facts create some space for speculation about future feasibility of studying N-BPs in terms of AD prevention/treatment. However, the possible impacts of N-BPs on Aβ generation and AD epidemiology remain to be determined.

This experimental study brings the first evidence of the inhibitive effect of N-BPs (alendronate) on cholesterol biosynthesis rates in different parts of rat CNS. Clinical significance of the described effects of these widely used agents on brain cholesterol synthesis ought to be resolved in further experiments and human studies.

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Abbreviations

Blood-brain barrier (BBB), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), Aβ (amyloid beta), acetylcholinesterase (AChE), Alzheimer´s disease (AD), central nervous system (CNS), fraction synthesis rate (FSR), nitrogen-containing bisphosphonates (N-BPs)

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Table 1: FSR (fraction synthesis rates) of cholesterol in various parts of the central nervous system

	Hippocampus	Basal ganglia	Frontal lobe	Spinal cord
Controls	0.048±0.0106	0.031±0.0075	0.056±0.0087	0.031±0.0087
Alendronate	0.035±0.0076***	0.030±0.0072	0.038±0.0077***	0.014±0.0084***

Legend: Results are expressed as mean ± standard deviation, symbol *** denotes p<0.001 vs. controls.