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Full Length Research Paper

Fluvastatin Reduces Amyloid Beta Production in Rat Brain by Modulating APP Processing and Secretase Expression

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The aim of the present study was to determine the effects of fluvastatin, a relatively hydrophilic 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) inhibitor, on endogenous amyloid beta (A) production and if such effects would be associated with changes in brain total cholesterol in rats. Wistar male rats were treated with fluvastatin at a dose of 20 mg/kg/day or vehicle (controls) by oral gavage for 28 days. We examined serum and brain cholesterol levels by CHOD-PAP method, brain A levels by radioimmunoassay, mRNA levels of HMGR and cholesterol 24S-hydroxylase (CYP46) involving in cholesterol balance, -secretase (BACE1) and -secretase (ADAM10) involving in amyloid beta precursor protein (A PP) processing by RT-PCR and protein levels of A PP by immunohistochemistry. Serum total cholesterol and brain A levels were significantly reduced in fluvastatin-treated rats. There was no change in total cholesterol levels, HMGR and CYP46 mRNA levels in the brain of fluvastatin-treated rats. Fluvastatin reduced A PP protein levels and up-regulated ADAM10 but down-regulated BACE1 mRNA expression in rat brain under used condition. These results suggest that reduction of brain A levels by fluvastatin is associated with changes in level of A PP and A PP cleavage-related enzyme mRNA, and is independent of brain total cholesterol. It may contribute to one of neuroprotective effects of fluvastatin and reveal that administration of fluvastatin could be beneficial in the preventation of AD.

Key words: Alzheimer's disease, cholesterol, amyloid , amyloid- protein precursor, fluvastatin, statin.

INTRODUCTION

Accumulation of amyloid beta (A) in the cortical and hippocampal regions of brain is one of pathological hallmarks of Alzheimer's disease (AD). A is generated from amyloid beta precursor protein (A PP) by sequential protease cleavage of -secretase (BACE1) and - secretase which aggregates to generate neurotoxic inso-luble A plaques. In alternative pathway, A PP is cleaved by -secretase (ADAM10) which cleaves within the A region to preclude A formation (Greenfield et al., 2000). Changes in cholesterol levels alter secretase cleavage of A PP as well as A levels. Higher levels of cholesterol promoted BACE1 activity to increase A production in neuron cells (Ghribi et al., 2006; Xiong et al., 2008); lower levels of cholesterol stimulated ADAM10 activity to decrease A production through the nonamyloidogenic pathway (Bodovitz and Klein, 1996; Kojro et al., 2001) demonstrating a close correlation between cholesterol and A production.

Statins are widely used drugs for treatment of hypercholesterolemia, and act to reduce serum cholesterol levels by inhibiting the rate-limiting enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), pre-venting de novo synthesis of cholesterol (Tobert, 2003). Epidemiological studies suggest that treatment with statins

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Abbreviations: Alzheimer's disease (**AD**); amyloid beta (**A**); amyloid beta precursor protein (**A PP**); -secretase (**ADAM10**); -secretase (**BACE1**); blood-brain barrier (**BBB**); cholesterol 24S-hydroxylase (**CYP46**); endothelial nitric oxide synthase (**eNOS**);3 -hydroxy-3-methylglutaryl-coenzyme A reductase (**HMGR**).

reduces the risk of developing AD (Wolozin et al., 2000; Rockwood and Darvesh, 2003). Experiment studies both *in vitro* and *in vivo* have also reported that treatment with statins attenuates A production in transgenic mouse (Refolo et al., 2001) and in cultural cells (Buxbaum et al., 2002) via up-regulating ADAM10 activity (Kojro et al., 2010) and down-regulating BACE1 activity (Sidera et al., 2005). The ability of statins to reduce cholesterol via inhibiting cholesterol synthesis has been suggested as a major mechanism for their anti-amyloidogenic property (Blain and Poirier, 2004).

It is well known that periphery and brain are strictly separated because of blood-brain barrier (BBB) in human and wild animals. Although there are two types of statins, lipophilic ones able to pass through BBB and hydrophilic ones which are not, the epidemiological studies showed both kinds of statins no difference in AD risk-reduction (Wolozin et al., 2000; Rockwood and Darvesh, 2003). Lipophilic simvastatin and lovastatin, and hydrophilic atorvastatin and fluvastatin were all reported to reduce endogenous A in brain of nontransgenic mice (Burns et al., 2006; Shinohara et al., 2010) as occurred in the brain of AD patients. Despite the recognized role for statins in A reduction, the accurate mechanisms are still not completely understood.

Fluvastatin is one of relatively hydrophilic statins. Studies have shown fluvastatin unable to cross BBB (Guillot et al., 1993) but effective in protecting BBB integrity (Kuhlmann et al., 2008) in nontransgenic mice. Consistent with these results, it was reported that fluvastatin decreased endogenous A levels through an increase in A clearance at BBB in nontransgenic mice (Shinohara et al., 2010) . In addition, thus far, there was no report that fluvastatin reduces brain cholesterol levels. In the present study, we therefore focused on the effects of fluvastatin on brain cholesterol and A levels in wide-type Wistar rats.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats were housed in cages with free access to standard rodent food and water and kept in a constant environment (22 ± 2°C, 50 ± 5% humidity and 12-h light/dark cycle). Animal experimental protocols followed the guidelines established by the Ethics Review Committee for Animal Experimentation (Hebei Medical University, Shijiazhuang, China).

Healthy Wistar male rats were provided by Experimental Animal Center of Heibei Medical University. Twenty rats (220 to 250 g) were divided randomly into control group (Con, n=10) and fluvastatin group (FV, n=10). Fluvastatin (Novartis, Switzerland) was suspended in 1% carboxymethyl cellulose and given by oral gavage to each rat at a final dose of 20 mg/kg/day for 28 d. The control group was given equivalent vehicle. 24 h after the final treatment, rats were sacrificed.

Tissue processing

Blood was collected and centrifuged to obtain serum for serum total cholesterol assay. Pieces of brains were submerged in liquid nitrogen and preserved at -70°C for extraction of total RNA and measurement of brain cholesterol and A levels. The rest pieces were separated into cortical and hippocampal regions, fixed in buffered formaldehyde and embedded in paraffin for A PP assay by immunohistochemistry.

Quantification of cholesterol

Cholesterol levels were determined by CHOD-PAP method. Serum total cholesterol levels were assayed using Olympus Au2700 full auto biochemical analyzer and cholesterol levels were adjusted to mmol/l.

To determine brain cholesterol content, total lipid in brain tissue was extracted according to the hexane/isopropanol method (Hara and Radin, 1978). The lipid extract was dried by nitrogen gas and solubilized in distilled water by the addition of triton X-100/isopropanol, cholesterol was then measured by CHOD-PAP method according to the instruction of kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The brain total cholesterol levels were adjusted to mg/10 g tissue.

Quantification of A by radioimmunoassay

Frozen brain tissue was homogenized in 10 volumes of cold physiological saline. The homogenate was centrifuged at 12000 g for 30 min at 4°C. The supernatant was assayed using A radio-immunity kit according to the manufacturer's recommendations (Beijing Puerweiye Biology and Technology Company Limited, Beijing, China). Radioactivity was measured in a -counter (FH-408, Beijing, China), and protein concentration was determined by Markwell modified Lowry assay. Brain A levels were adjusted to pg/mg protein.

Analysis of RNA expression by RT-PCR

The relative mRNA levels were measured by semi-quantitative RT-PCR using the housekeeping gene -actin as normalization control. Total RNA was isolated using the TRIzol reagent (Invitrogen, USA). cDNA was synthesized from 1 g of total RNA using promega reverse transcription system (Promega, USA) according to the manufacturer's protocol. PCR was performed in Promega PCR System using primers as follows: 5'-TGT CAT TCC AGC CAA GGT G-3' and 5'-GCC CGT GTT TCA GTT CAA GTA-3' (570 bp) for HMGR; 5' -TGA GAC TTC CGC CAA CAA T-3' and 5'- CAC CTC CAT CTG AGC AAA CTG-3' (430bp) for CYP46; 5' -TCC CAA GCC CAA CTT TAC-3' and 5'-ACC AGT GAG CCA CAA TCC-3' (395 bp) for ADAM10; 5'- GGC GGG AGT GGT ATT ATG AA-3' and 5'- GTG ATG CGG AAG GAC TGA TT-3' (316 bp) for BACE1; 5'- CAT CCT GCG TCT GGA CCT- 3' and 5'-TCA GGA GCA ATG ATC TTG-3'(480bp) for -actin respectively. The PCR products were seperated on 1.5% agarose gel, stained with ethidium bromide, and visualized using the JD801 imaging analysis system (Jiangsu JEDA Science-Technology Development Company, Limited, Nanjing, China). Each mRNA level was normalized to -actin mRNA.

Analysis of A PP by Immunohistochemistry

The avidin-biotin-peroxidase complex method was performed on deparaffinized sections to detect the levels of A PP using diaminobenzidine (DBA) as chromogens. Briefly, tissue sections were sequentially incubated with primary antibody against A PP (6E10, Abcam, Japan), biotinylated secondary antibody, streptavidin-horseradish peroxidase and DAB (Beijing Zhong

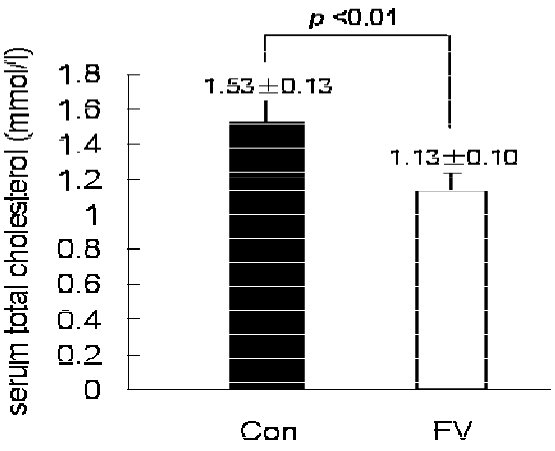

Figure 1. Total cholesterol levels in serum of vehicle-treated (Con) and fluvastatin-treated (FV) rats.

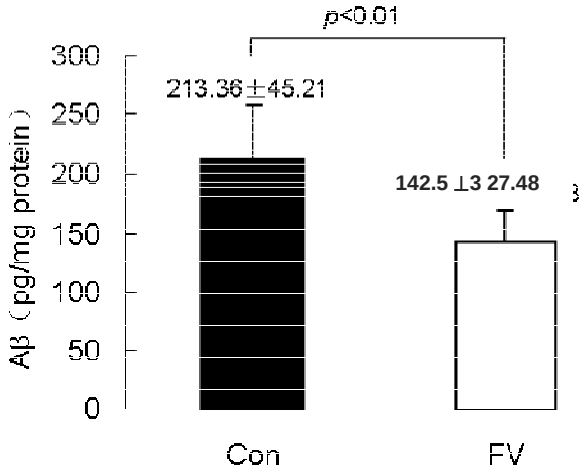

Figure 2. Total A levels in brain of vehicle-treated (Con) and fluvastatin-treated (FV) rats.

Shan -Golden Bridge Biological Technology Company Limited, Beijing, China) for developing.

Statistical analysis

All results are presented as mean ± SD. Standard statistical analyses were performed via student's *t*-test in SPSS 10.0. Statistical significance was set at $p<0.05$.

RESULTS

Fluvastatin significantly reduces serum total cholesterol

A significant decrease in serum total cholesterol was found in fluvastatin-treated rats (FV, 1.13±0.10 mmol/L) compared with vehicle-treated controls (Con, 1.53±0.13 mmol/L, p<0.01) (Figure 1 and Table 1).

Fluvastatin significantly reduces endogenous brain A levels

Measurement of total A in brain homogenates by radioimmunoassay showed an approximate 33% (p<0.01) decrease in A in fluvastatin-treated rats compared with vehicle-treated ones (Figure 2).

Fluvastatin has no effects on brain cholesterol levels

Cholesterol homeostasis in brain is maintained by balancing de novo synthesis, transporter efflux and conversion of cholesterol into 24S-hydroxycholesterol by cholesterol 24S-hydroxylase (CYP46) (Heverin et al., 2004). In addition, HMGR may be feedback regulated at transcription and blocked by potent inhibitors, statins (Istvan, 2002). Therefore, changes in mRNA levels of HMGR and CYP46 gene can also reflect alterations in cholesterol content (Locatelli et al., 2002). No changes were observed in cholesterol (Figure 3A) and HMGR and CYP46 mRNA levels (Figure 3B) in brain of fluvastatin-treated rats by spectrophotometry and RT-PCR respectively.

Alteration in expression of ADAM10, BACE1 and A PP

Changes in A levels were postulated to result from changes in the activity of - and -secretase affected by intracellular cholesterol levels (Puglielli et al., 2003). Since no changes in cholesterol levels were found in the present study, we observed the expression of ADAM10, BACE1 at mRNA levels and A PP at protein levels in brain to determine the cause of A reduction by fluvastatin. Results showed that the mRNA expression of ADAM10 was up-regulated while BACE1 mRNA expression was down-regulated (Figure 4) and the amount of A PP in cortex and hippocampus was significantly reduced in fluvastatin-treated rats (Figure 5).

DISCUSSION

Clinical and epidemiological data suggest that both lipophilic and hydrophilic statins administration could be of benefit in decelerating the incidence of AD with decreased serum cholesterol (Rockwood et al., 2002). The statin-decreased serum cholesterol was accompa-nied with lowed brain A levels in guinea pigs (Fassbender et al., 2001), transgenic (Tong et al., 2009) and non-transgenic mice (Burns et al., 2006; Shinohara

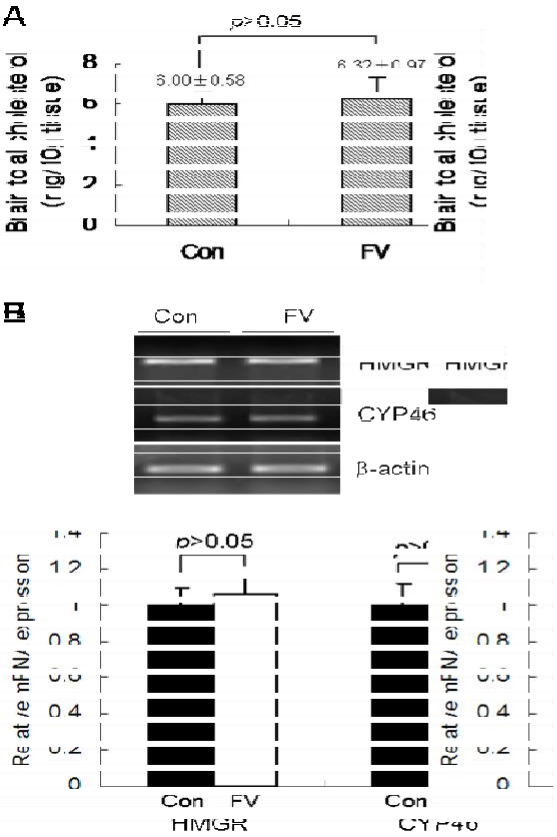

Figure 3. Levels of total cholesterol (A) and HMGR and CYP46 mRNA (B) in brain of vehicle-treated (Con) and fluvastatin-treated (FV) rats. The top panel in B illustrates the electrophoretic results of RT-PCR and the lower panel depicts the corresponding quantitative data (n=10 each group).

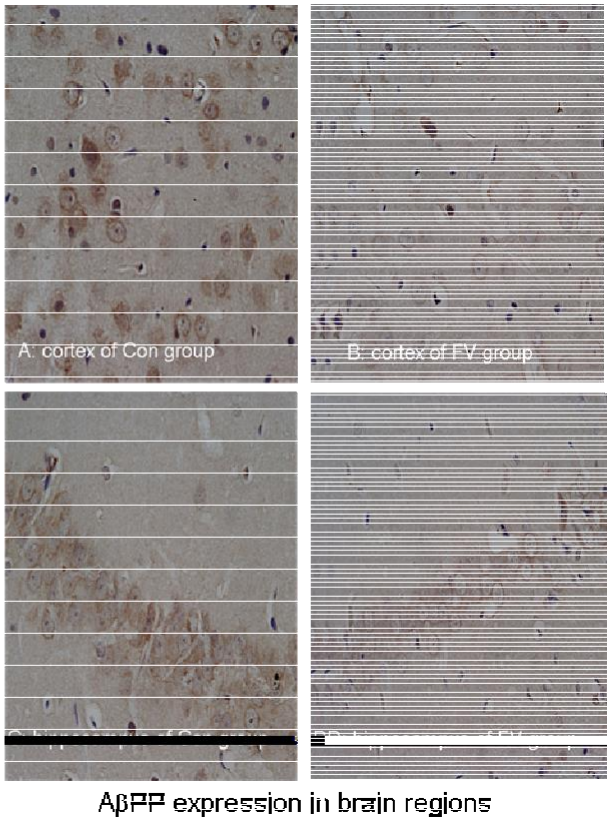

Figure 5. Levels of A PP in cortex and hippocampus of rats by immunohistochemistry. A and C: control group; B and D: fluvastatin treated group (DAB brown staining, original magnification ×400).

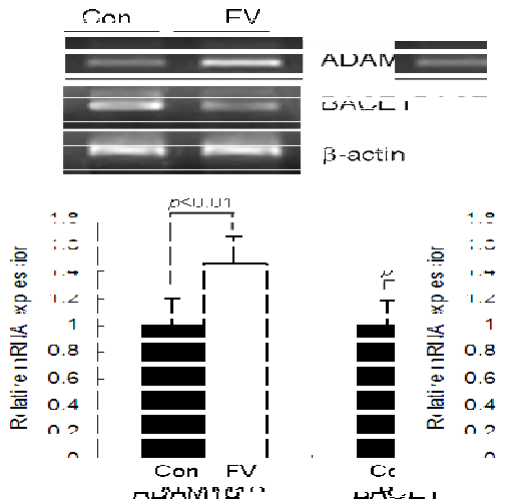

Figure 4. Levels of BACE1 and ADAM10 mRNA in brain of vehicle-treated (Con) and fluvastatin-treated (FV) rats. The top panel illustrates the electrophoretic results of RT-PCR and the lower panel depicts the corresponding quantitative data (n=10 each group).

et al., 2010). Consistent with these reports, we observed that hydrophilic fluvastatin significantly reduced serum cholesterol and endogenous brain A levels in rats. Our data indicated that fluvastatin, in addition to its lipid-lowering effects in periphery tissues, has other protective effects in prevention of AD.

However, fluvastatin had no effect on brain total cholesterol levels in current studies although it obviously reduced brain A in rats. It is supported by the previous studies that lipophilic simvastatin, lovastatin, and hydropholic atorvastatin all reduced endogenous A levels and only lovastatin reduced brain total cholesterol levels in non-transgenic mice (Burns et al., 2006). Furthermore, we found there was no alteration in mRNA expression of HMGR and CYP46 genes with suspected roles in synthesis and turnover of cholesterol respectively in the brain of fluvastatin-treated rats, suggesting that brain cholesterol levels and cholesterol metabolism are not modulated by fluvastatin. These results support the substantial studies that statins reduce brain A levels in brain total cholesterol level-independent manner (Burns et al., 2006; Thelen et al., 2006). Because cholesterol and cholesterol synthesis intermediates and derivatives are very important in the brain, hydrophilic statins which

can not pass through the BBB could be more secure (Fonseca et al., 2010). Based on our data, fluvastatin could be one of more secure statins.

The proteolytical cleavage of A PP by BACE1 generates A . The cleavage of A PP by ADAM10 precludes A generation. The increased A PP and BACE1 but decreased ADAM10 expression seems to be associated with the formation of amyloid plaques in the brains of AD patients (Rossner et al., 2006; Colciaghi et al., 2002). Consistent with these studies, we found that decreased endogenous A levels were accompanied with the decreased levels of A PP protein, decreased levels of BACE1 mRNA, and increased of ADAM10 mRNA in brain following a decrease in serum cholesterol in fluvastatin-treated rats. This study suggests that the effect of fluvastatin on brain A metabolism in rats might not be through cholesterol-mediated changes in activity of A PP-cleaving enzymes but through changes in expression of A PP, BACE1 and ADAM10.

Relatively hydrophilic fluvastatin has been shown unable to pass through the BBB (Guillot et al., 1993) but effective in protecting BBB integrity (Kuhlmann et al., 2008). In addition, it was reported that high doses of lipophilic simvastatin (100 mg/kg/day) crossed BBB to affect brain cholesterol synthesis in mice administrated for 3 days, but high doses of hydrophilic pravastatin (200 mg/kg/day) failed to do so (Thelen et al., 2006). Here, we treated normal rats with low dose (20 mg/kg/day) of fluvastatin for 28 days. Therefore, it might be the possibility that without entering into the brain, fluvastatin indirectly reduced brain A levels in a non-cholesterol-lowing manner under used conditions (Cheng et al., 2010; Lu et al., 2010). However, it is still unclear by which mechanism fluvastatin regulated the expression of A PP and A PP-cleaving enzymes, ultimately resulted in the reduction of brain A levels without entering into the brain. It was reported that fluvastatin decreased endogenous A levels through an increase in A clearance at BBB in nontransgenic mice (Shinohara et al., 2010). Besides, reduction of NO levels by inhibiting the enzyme catalyzed NO biosynthesis, endothelial nitric oxide synthase (eNOS), or by knocking eNOS gene out led to increased A PP and BACE1 protein levels, as well as increased secretion of A in brain microvascular endothelial cells (Austin et al., 2010). Furthermore, fluvastatin was recently reported to phosphorylate and activate eNOS, and increase eNOS expression, thereby increasing NO production in vascular endothelial cells (Aoki et al., 2010). Therefore, it might be speculated that the final central effects of fluvastatin are initiated indirectly at BBB micro vessel wall, e.g. by up-regulation of eNOS, subsequently increased NO crosses BBB to modulate A PP expression and process within the brain.

Conclusively, the present studies show the following findings in rats: 1. fluvastatin obviously reduced brain A levels without altering brain total cholesterol levels; 2. fluvastatin reduced A PP protein levels and up-regulated ADAM10 but down-regulated BACE1 mRNA expression.

Reduction of brain A levels by fluvastatin is associated with changes in levels of A PP and mRNA of genes involved in A PP cleavage and is independent of brain total cholesterol. It may contribute to one of neuroprotective effects of fluvastatin and reveal that administration of fluvastatin could be beneficial in the prevention of AD.

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