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Increase of Amyloid-Beta Peptide Generation in High Cholesterol Diet Rabbit Brain

Yong Kyoung LEE[1], Dong Ju SON[1], Jae Woong LEE[1,2], Hyung Woo LEE[1], Young Won YUN[3], Ki-Wan OH[1], and Jin Tae HONG[1,2]*

[1]College of Pharmacy and [2]CBITRC, [3]College of Veterinary Medicine, Chungbuk National University, 12 Gaeshin-dong, Heungduk-gu, Cheoungju, Chungbuk 361-763, Korea

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Abstract – Alzheimer's disease (AD) is an abnormal accumulation of the β-amyloid protein (Aβ) in specific brain region. It has been speculated that disturbance in cholesterol homeostasis may contribute to the etiology of AD by increasing Aβ generation. However, conclusive evidence and possible mechanism has not been reported. In the present study, we demonstrated that rabbits treated with 0.5% cholesterol for 16 weeks increased serum total cholesterol, triacylglycerol, and low-density lipoprotein levels. Aβ levels is higher in the hippocampus of brain in cholesterol dieted rabbits than that of normal diet rabbis. Expression and activities of β- and γ- secretases, the enzymes that cleave β-amyloid precursor protein to generate Aβ, were also increased in hippocampus of high cholesterol dieted rabbit than those of normal dieted rabbits. Our results suggest that high cholesterol diet may be associated with increased Aβ accumulation in the brain of rabbits, and suggest that high cholesterol diet may be causal factor in the development or progression of AD.

Key words □ Alzheimer's disease, high cholesterol diet, beta-amyloid, β- and γ- secretase, rabbit

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative process characterized by loss of memory, cognition, and behavioral stability. AD is defined pathologically by accumulation of extracellular neuritic plaque comprised of fibrilllar deposits of amyloid β-peptide (Aβ) and neurofibrillary tangles comprised of paired helical filaments of hyperphosphorylated tau (Hardy *et al.*, 2002).

Epidemiological and animal studies suggest that disturbances in cholesterol homeostasis may contribute to the etiology of AD (Fassbender *et al.*, 2001; Galbete *et al.*, 2000; Jick *et al.*, 2000; Racchi *et al.*, 1997; Refole *et al.*, 2000). Epidemiological studies have shown that people in the UK who are prescribed statins, cholesterol lowering agent (lipid-lowering agents: LLAs), have a 37%~70% high risk of dementia than those who do not have hyperlipidaemia or who are not treated with LLAs (Jick *et al.*, 2000). In animal studies, dietary cholesterol accelerates Aβ depostion in the brain of transgenic mice expressing familial AD mutant, human APP $_{K671N,M6711}$ (Refole *et al.*, 2000). BM15.766, a cholesterol-lowing drug, reduces Aβ depositon in the brain of AD and in the brain of APP $_{K671N,M6711}$ mutant mice (Refole *et al.*, 2001). Although high cholesterol can influence on Aβ generation, it is note worthy that a change in membrane properties, including stiffness and fluidity, has been suggested to influence activities of membrane-bond proteins and enzymes, including secretase. Cellular cholesterol content is increased in neuron by cholesterol enriched diet (Othman *et al.*, 2006). Thus, the high cholesterol content in lipid rafts, membrane regions where these enzymes are located, facilitates the clustering of the secretases with their substrates into an optimum configuration, promoting the undesirable pathogenetic cleavage of amyloid precursor protein (Bodovitz *et al.*, 1996; Witter *et al.*, 1991). However, direct relationship between high cholesterol diet and Aβ depositon, and the mechanism by which cholesterol affects Aβ production and metabolism is not fully understood.

Because 2% cholesterol diets cause severe hypercholesterolemic side effects, requiring sacrifice of the cholesterol-fed rabbits at 8 weeks (Sparks *et al.* 1994; Sparks *et al.* 2000), we have anticipated that a long-term diet supplemented with a low level of cholesterol would allow the animals to live longer, thus

*Corresponding author
Tel: +82-43-261-2813, Fax: +82-43-268-2732
E-mail: jinthong@chungbuk.ac.kr

facilitating the accumulation of $A\beta_{1-42}$ and the activation of enzyme involved in Aβ generation. In present study, we have examined the effect of 0.5% cholesterol-enriched diet on levels of serum cholesterol, beta-amyloid, and activation of enzyme involved in Aβ generation in rabbit.

MATERIALS AND METHODS

Hypercholesterolemia animal model

New Zealand white female rabbits were used for the proposed studies. Animals were randomly assigned to 2 groups as follows: group 1, normal chow (n=3) and group 2, chow supplemented with 0.5% cholesterol (n=5). The animals were dieted for 16 weeks. Diets were kept frozen at -10°C to reduce the risk of oxidation. Cholesterol-dieted animals and their matched controls were euthanized 16 weeks later. At necropsy, each brain was immediately frozen in liquid nitrogen, placed into a zipper-closure plastic bag, and buried in dry ice pellets until transferring to -80°C for storage before sectioning, western blot, and Aβ assay.

Measurement of serum cholesterol level

Total serum cholesterol and triglycerides were determined enzymatically as previously described (Haubenwalner *et al.*, 1995). Serum lipoprotein cholesterol profiles and distribution among lipoproteins were determined by on-line post column analysis on Superose 6HR high performance gel filtration chromatography (HPGC). Lipoprotein cholesterol was determined by multiplying the independently determined total serum cholesterol by the percent area for each lipoprotein distinctly separated by the HPGC method.

Measurement of brain Aβ level

The Aβ level in brain was determined using available $A\beta_{1-42}$ assay kit (Immuno-Biological Laboratories Co., Aramachi, Japan) according to the manufacture's protocols. The whole brain, cerebral cortex and hippocampus were homogenized in protein extraction solution (PRO-PREP™, Intron Biotechnology, Korea). To determine Aβ, 100 ml lysate was put in 96 well plate which was coated with anti-Human Aβ (38-42) rabbit lgG and the plate was incubated for 12 hr at 4°C. 100 μl labeled antibody solution was added into the wells and followed by incubation 1 hr at 4°C in the dark. Tetra Methyl Benzidine (TMB) was added each well then the plate was incubated for 30 minutes for room temperature in the dark. TMB is used as coloring agent (Chromogen). The reaction was stopped by addition of 100 ml stop solution (1N H_2SO_4). The Aβ amount was quantified by measured light absorbance at 450 nm within 30 minutes after application of stop solution. The Aβ amount was linearly related to the increase in light absorbance. The Aβ amount was expressed as pg produced substrate which was determined by the formation of pg per mg protein.

Measurement of β- and γ- secretase activity in brain

The total activities of β- and γ-secretase present in cortical and hippocampal region which were determined using commercially available β-secretase fluorescence resonance energy transfer (BACE 1 FRET) assay kit (PANVERA, Madison, USA) and g-secretase activity Kit (R&D systems, Wiesbaden, Germany) according to the manufacture's protocols, respectively. The cerebral cortex and hippocampus were homogenized in cold 1× cell extraction buffer (ready for use in the kit) to yield a final protein concentration of 1 mg/ml.

To determine β-secretase, 10 μl lysate was mixed with 10 μl BACE1 substrate (Rh-EVNLDAEFK-Quencher), and then the reaction mixture was incubated for 1 hr at room temperature in the 96 well flat bottom microtitre plate. The reaction was stopped by addition of 10 μl BACE1 stop buffer (2.5 M sodium acetate). The formation of fluorescence was read with Fluostar galaxy fluorometer (excitation at 545 nm and emission at 590 nm) with Felix software (BMG Labetchnologies, Offenburg, Germany). The enzyme activity was linearly related to the increase in fluorescence. The enzyme activity was expressed as nM produced substrate which was determined by the formation of fluorescence per mg protein per min.

To determine γ-secretase, 50 μl lystate was mixed with 50 μl reaction buffer. The reaction mixture was then incubated for 1 hr in the dark at 37°C. 5 ml substrate was added after the periods of incubation time to stop the reaction. Cleaved substrate by γ-secretase was conjugated to the reporter molecules EDANS and DABCYL, and released fluorescent signal. This formation of fluorescence was read with Fluostar galaxy fluorometer (excitation at 355 nm and emission at 510 nm) with Felix software (BMG Labetchnologies, Offenburg, Germany). The level of g-secretase enzymatic activity is proportional to the fluorometric reaction, and the g-secretase activity was expressed the produced fluoresce unit. All controls, blanks and samples were run in triplicate.

Western blotting

Brains were homogenized with lysis buffer and centrifuged at 23,000 g for 1hr. The protein concentration was measured by

the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories Inc, Hercules, CA), and equal amount of proteins (20 μg) were separated on a SDS/10%, 15% and 20% -polyacrylamid gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 1 hr at room temperature with 5 % (w/v) non-fat dried milk in Tris-buffered saline. The membrane was incubated for 5 hr at room temperature with specific antibodies: APP (1:1000), BACE (1:1000), C99 (1:1000) and β-actin (1:10000) as internal standard. Immunoreactive proteins were detected with the ECL western blotting detection system.

RESULTS

Serum cholesterol level in high cholesterol dieted rabbit

Examination of the lipid profiles of high cholesterol diet versus control rabbits reveals a significant elevation in the amount of total cholesterol, triacyglycerol and low density lipoprotein in the high cholesterol diet rabbits (Table I). Serum total cholesterol concentrations 43.00 ± 9.29 mg/dl in controls and this level was elevated to 1118.57 ± 126.90 mg/dl in high cholesterol dieted rabbits. Triacylglycerol level was 15.33 ± 2.33 mg/dl in normal whereas 37.17 ± 7.11 mg/dl in high cholesterol dieted rabbits. However, HDL level did not show significant changed (15.67 ± 2.03 mg/dl vs 21.21 ± 10.21 mg/dl). Especially, serum LDL level in cholesterol diet rabbits (381.43 ± 167.23 mg/dl) was increased over 10 times than controls (36.33 ± 19.14 mg/dl).

$A\beta_{1-42}$ content and secretase activity in the brain of high cholesterol dieted rabbits

We used Aβ assay kit to measure $A\beta_{1-42}$ content in whole brain, cortex and hippocampus. $A\beta_{1-42}$ level was increased in the whole and hippocampus of cholesterol-treated rabbit. However, $A\beta_{1-42}$ levels was not changed in cortex of cholesterol dieted rabbits (Fig. 1). Since Aβ is produced with serial cleavage of amyloid precursor protein (APP) by β- and γ- secretase. We determined secretase activity in brain. Consistent with the elevated levels of Aβ, β- and γ- secretase activities were increased in whole, and cortex and hippocampus of high cholesterol diet rabbits (Fig. 2).

Table I. Cholesterol concentrations in serum of control and cholesterol diet rabbits

	Control (n=3)	Cholesterol (n=14)
Total cholestrol (mg/dl)	43.00±9.29	1118.57±126.90
Triacylglycerol (mg/dl)	15.33±2.33	37.14±7.11
High-denstiy lipoprotein (mg/dl)	15.67±2.03	21.21±10.21
Low-density lipoprotein (mg/dl)	19.67±7.36	381±44.70

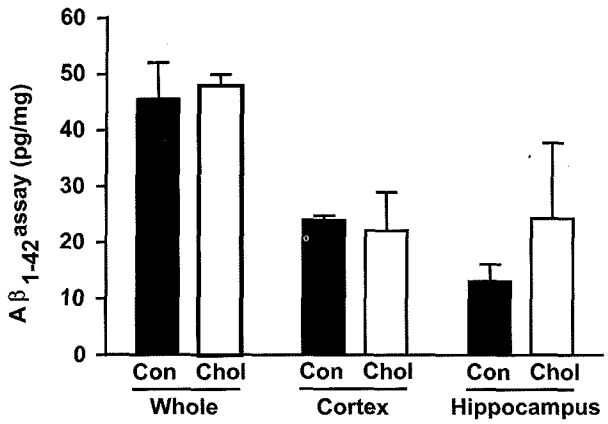

Fig. 1. $A\beta_{1-42}$ level in the brain of control and high cholesterol diet rabbit. High cholesterol diet rabbit were served 0.5% cholesterol contained food for 16 weeks. $A\beta_{1-42}$ level was measured by commercial Aβ assay kit in the whole brain, hippocampus and cortex of control (con) and high cholesterol diet (chol) rabbit.

APP processing change in the brain of cholesterol dieted rabbits

Western blotting was next done to determine whether APP processing could be altered in cholesterol-dieted rabbit brain. Consistent with the increase of β- and γ- secretase activity, western blot analysis showed that β-APP, BACE and its C-terminal fragment, C99, which is the product of cleavage of β-APP by β-secretase (BACE) levels were increased in whole brain and hippocampus region of high cholesterol diet rabbits. However, β-APP, BACE and C99 protein levels were not sighificantly changed in cortex of high cholesterol diet rabbits (Fig 3).

DISCUSSION

In the present study, we have examined the relationship between hypercholesterolemia and Aβ generation using high cholesterol dieted rabbit model. We severed food contained 0.5 % cholesterol to rabbit for 16 weeks. In results, serum low density lipoprotein (LDL) and triacyglyceride levels were significantly increased in high cholesterol dieted rabbits. Aβ level was

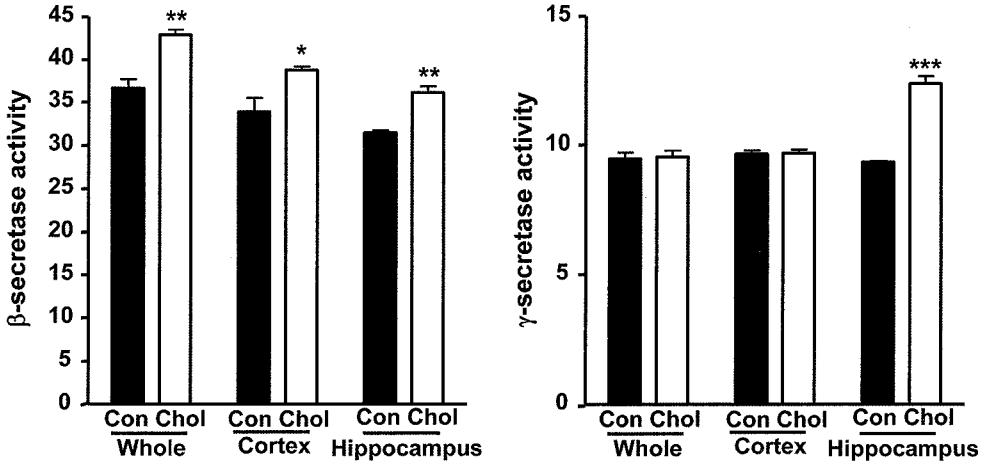

Fig. 2. β- and γ- secretase activities in the brain of control and high cholesterol diet rabbit. β- and γ- secretase activities were measured by secretase assay kit in the whole brain, cortex and hippocampus of control (con) and high cholesterol diet (chol) rabbit. High cholesterol diet rabbit was made by serving 0.5% cholesterol contained food for 16 weeks. *P<0.05 indicates statistically significant differences between control and high cholesterol diet rabbit.

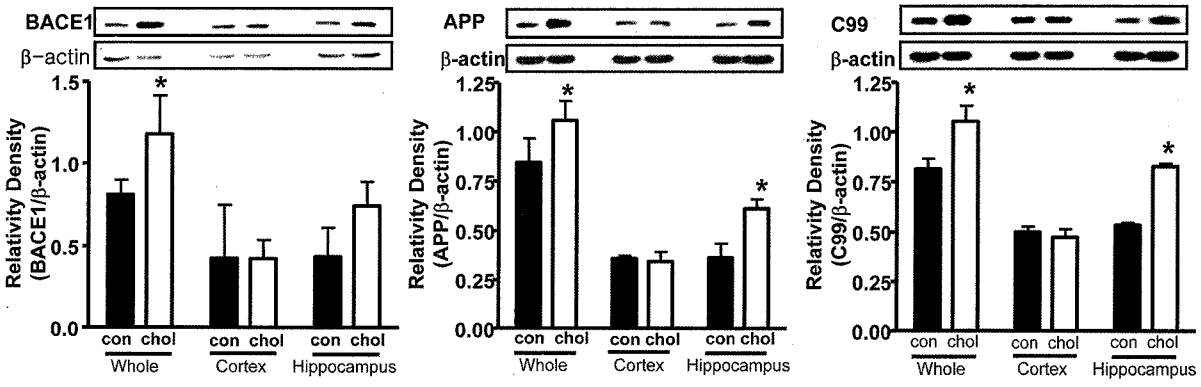

Fig. 3. Expression of BACE1, APP and C99 in the brain of control and high cholesterol diet rabbit. High cholesterol diet rabbit was served 0.5% cholesterol contained food for 16 weeks. The brains of control (con) and high cholesterol diet (chol) rabbit were used for western blot. Equal amounts of total proteins (40 mg/lane) were subjected to 6% (APP), 10% (BACE1) and 15% (C99) SDS-PAGE. Expression of BACE1, APP, C99 and β-actin were detected by western blotting using specific antibody. β-actin protein was used as an internal control. *P<0.05 indicates statistically significant differences between control and high cholesterol diet rabbit.

increased in high cholesterol dieted rabbit brain. In addition, we also found that β- and γ- secretase activities were increased in brain of high cholesterol diet rabbits accompanied by the increased expression of BACE, APP and C99 which these proteins were involved in Aβ generation.

A number of epidemiological studies suggest that high levels of dietary cholesterol may contribute to the pathogenesis of AD. AD patients have increased levels of total serum and low-density lipoprotein (LDL) cholesterol along with reduced levels of high-density lipoprotein (HDL) in their plasma, as compared to age-matched controls (Fernandes., 1999; Kuo *et al.*, 1998). Cholesterol abnormally accumulates in the dense cores of amyloid plaques in the brain of AD patients (Mori., 2001). Similar accumulation of cholesterol has also been found in amyloid plaques of transgenic mice expressing a mutant form APP_{695} (Swedish mutation) associated with FAD (Mori., 2001). Studies of using transgenic animal models of AD show a strong connection between plasma cholesterol levels and Aβ generation (Fassbender *et al.*, 2001; Holsinger., 2002; Refolo *et al.*, 2000). A recent report shows that high-cholesterol diet containing 5% cholesterol, 10% fat, and 5.2 kcal/g for 7 weeks raise cholesterol levels in plasma and CNS of transgenic mice expressing the FAD mutant $APP_{K670,M671L}$ and $PS1_{M146V}$. Both β-APP C-terminal fragment (CTF) and Aβ levels were

increased in the brain of these animals (Refolo *et al.*, 2000). The result suggested that high cholesterol diet may be implicated in the development of AD. Other in vitro studies also have shown that a high cholesterol environment results in reduced production of soluble amyloid precursor protein (Kojro *et al.*, 2001; Racchi *et al.*, 1997; Simons *et al.*, 1998; Wahrle *et al.*, 2002), a protein protecting neurons against cell stimuli including Aβ. Neuropathological analysis showed that a high cholesterol diet also increased the deposition of amyloid plaque in presenilin 1 transgenic mice, AD model mice (Holcomb *et al.*, 1998; McGowan *et al.*, 1999). Additionally, cholesterol-lowering agents reversed the effect of high fat/high cholesterol diet on Aβ accumulation and cholesterol levels in the plasma and CNS (Refolo *et al.*, 2001). Taken together these data suggest that high cholesterol diet may increase of Aβ level in the brain, thereby affect AD development.

The mechanism by which dietary cholesterol promotes Aβ peptide accumulation is not fully understood. However, cholesterol influences the activities of the enzymes involved in the metabolism of the amyloid precursor protein to generate Aβ. The post-translational cleavage of amyloid precursor protein by β- and γ- secretase, membrane associated proteins, results in amyloidogenic products that aggregate as extracellular plaques (Leila *et al.*, 2005). β-secretase activity has been demonstrated to be increased in lipid microdomains (Cordy *et al.*, 2003; Marlow *et al.*, 2003), and is upregulated in sporadic cases of Alzheimer's disease (Holsinger *et al.*, 2002; Yang *et al.*, 2003). Lipid microdomains that are enriched in cholesterol are critically involved in the initial cleavage of APP by β-secretase to generate Aβ (Ehehalt *et al.*, 2003). The cholesterol-induced increase in APP and BACE is associated with an increase in C99 levels. Therefore these results suggest that β-secretase and APP processing are upregulated by the cholesterol diet, resulting in an increase in the cleavage of APP to C99 and Aβ peptide.

It is interested to know that Aβ content was significantly accumulated in hippocampus compared with cortex region by 0.5% cholesterol diet in rabbit brain. A variety of memory task has been known that hippocampus is specifically involved in memory (Mishkin., 1978; Squire *et al.*, 1991). In addition, more recent reports have indicated that subjects with hippocampal damage can exhibit impaired memory (Simons *et al.*, 1998). Even though the hippocampal dentate gyrus receives a direct excitatory input from the ipsilateral entorhinal cortex (Witter *et al.*, 1991), an area known to be affected early in course of Alzheimer disease (Gomez-Isla *et al.*, 1996; Van Hoesen *et al.*, 1991), one of the major neuropathological findings in the brains of individuals with AD is loss of synaptic contact in hippocampus (Bertoni-Freddari *et al.*, 1990; Bodovitz *et al.*, 1996; Goto *et al.*, 1990; Hamos *et al.*, 1989; Masliah *et al.*, 1989). The accumulation of the loss of synapses and a failure to replace the loss synaptic contacts in hippocampus may lead to the decline in cognitive function that is clinically assessed as a decline in memory. Thus, high cholesterol diet could affect on the memorial function of hippocampus via accumulation of Aβ.

The present study showing high cholesterol diet increase Aβ generation, and the elevated Aβ is associated with increase β- and γ- secretase activities and with the increase of the express of BACE, APP and C99 suggest that high cholesterol diet could contribute to the development or progression of AD.

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