

Hypercholesterolemia accelerates amyloid β -induced cognitive deficits

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Abstract. Hypercholesterolemia is a known risk factor for Alzheimer's disease (AD). In the present study, we investigated whether diet-induced hypercholesterolemia affects AD-like pathologies such as amyloid β -peptide ($A\beta$) deposition, tau pathology, inflammation and cognitive impairment, using an $A\beta_{25-35}$ -injected AD-like pathological mouse model. Hypercholesterolemia was induced by providing apolipoprotein E knock out (Apo E KO) mice with a high-fat diet for 4 weeks prior to $A\beta_{25-35}$ injection and for 4 weeks following $A\beta_{25-35}$ injection, for a total of 8 weeks of treatment. Our data showed that intracerebroventricular injection of C57BL/6J mice with $A\beta_{25-35}$ resulted in increased immunoreactivity of $A\beta$ and phosphorylated-tau (p-tau), which was accompanied by enhanced microglial CD11b-like immunoreactivity in the brain. Moreover, hypercholesterolemia slightly increased $A\beta$ and p-tau levels and microglial activation in the vehicle group, while further increasing the $A\beta$ and p-tau levels and microglial activation in $A\beta_{25-35}$ -injected mice. Consistent with the neuropathological analysis, hypercholesterolemia resulted in significant spatial learning and memory deficits in $A\beta_{25-35}$ -injected mice as revealed by water maze testing. Collectively, these findings demonstrated that hypercholesterolemia accelerated $A\beta$ accumulation and tau pathology, which was accompanied by microglial activation and subsequent aggravation of memory impairment induced by $A\beta_{25-35}$. Thus, we suggest that the modulation of cholesterol can be used to reduce the risk of developing AD.

Introduction

Alzheimer's disease (AD) is characterized by cerebrovascular damage and neuronal dysfunction leading to progressive cognitive decline. The hallmark pathologies of AD include amyloid β -peptide ($A\beta$) deposition, neurofibrillary tangles containing hyperphosphorylated tau and increased microglial reactivity as well as inflammatory processes. Hypercholesterolemia, a well-established vascular risk factor, is associated with the pathophysiology of AD (1,2) and it has also been shown to increase the production of $A\beta$ in animal and cellular models (3-7). However, the mechanism by which hypercholesterolemia causes $A\beta$ accumulation in the brain and contributes to the pathogenesis of AD remains unclear.

Hypercholesterolemia and AD share inflammation as a pathophysiological trait. It was considered that high levels of serum cholesterol would induce vascular changes similar to early inflammatory lesions of atherosclerosis and blood brain barrier permeability as well as localized neuroinflammatory changes (8). Furthermore, chronic inflammatory responses including microglial activation have been detected in AD brains (9,10). It is likely that hypercholesterolemia resulting from either dietary practice or genetic disposition facilitates an exacerbated neuroinflammatory response in association with increased $A\beta$ generation, leading to AD-associated degeneration. To test this theory, we administered a high-fat diet to a hypercholesterolemic strain (Apo E KO mice) for 4 weeks prior to $A\beta_{25-35}$ injection and for 4 weeks following $A\beta_{25-35}$ injection, for a total of 8 weeks of treatment. This was followed by assessment of cognitive function with respect to neuroinflammatory and AD-related biochemical changes.

We hypothesized that hypercholesterolemia accelerates amyloid pathology, tau pathology and neuroinflammation, subsequently exacerbating the cognitive decline in AD. We investigated the effects of hypercholesterolemia on AD-related neuropathological markers including $A\beta$, phosphorylated-tau (p-tau) and microglial activation in the brains of normal and hypercholesterolemic mice injected with $A\beta_{25-35}$ (a toxic fragment of full-length $A\beta$). Spatial learning and memory in $A\beta_{25-35}$ -injected mice was assessed by the Morris water maze test. Exploring the interplay between hypercholesterolemia and AD may enable development of a rational basis for the prevention and treatment of AD underlying clinically detected dementia.

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Materials and methods

A β_{25-35} injection model. Wild-type male C57BL/6J and apolipoprotein E knock out (Apo E KO) mice (Japan SLC, Shizuoka, Japan) were housed under diurnal lighting conditions and allowed food and tap water *ad libitum*. All animal procedures were conducted in accordance with the institutional guidelines for animal research, and approved by the University Animal Care and Use Committee (PNU-2011-000419). An A β -induced cognitive impairment mouse model was produced based on modification of previously reported methods (11). Anesthesia was achieved by isoflurane (2% induction and 1.5% maintenance, in 80% N₂O and 20% O₂) administered via a face mask. The depth of anesthesia was confirmed by the absence of cardiovascular changes in response to tail pinch. Rectal temperature was kept at 36.5–37.5°C using a Panlab™ thermostatically controlled heating mat (Harvard Apparatus, Holliston, MA, USA). A β_{25-35} (10 nmol in 5 μ l of saline; Sigma-Aldrich, St. Louis, MO, USA) was injected intracerebroventricularly (icv) into the mice, aimed at 1 mm lateral to the midline, 0.5 mm posterior to the bregma and 3 mm deep using a 25 μ l Hamilton syringe with a 26-gauge needle (Hamilton, Reno, NV, USA) at a rate of 0.5 μ l/min using a stereotaxic injector (KD Scientific, Holliston, MA, USA). The vehicle group of mice received icv injections of an equal volume of saline. Hypercholesterolemia was induced by providing a Western-type high-fat diet (42% of total calories from fat; 0.15% cholesterol; Research Diets, New Brunswick, NJ, USA) for 4 weeks in Apo E KO mice prior to A β_{25-35} injection and for 4 weeks following A β_{25-35} injection, for a total of 8 weeks.

Measurement of plasma cholesterol. Blood was collected from the left ventricle under light anesthesia and stored on ice for 30 min prior to centrifugation at 13,000 rpm and 4°C for 10 min, after which the plasma was separated and kept at -80°C until assayed. The lipoprotein cholesterol distribution of plasma samples was determined enzymatically with reagents from Kyowa Medex (Tokyo, Japan) using a TBA-200FR NEO apparatus (Toshiba Medical Systems, Tokyo, Japan).

Immunohistochemistry. Four weeks after A β_{25-35} injection, mice were deeply anesthetized with thiopental sodium and subsequently perfused transcardially with cold PBS followed by 4% paraformaldehyde for fixation. The brain of each mouse was then removed and further fixed for 24 h in 4% paraformaldehyde at 4°C followed by cryoprotection in 20% sucrose for 72 h at 4°C. Next, the isolated brains were frozen in an optical cutting temperature medium for frozen tissue specimens (Sakura Finetek, Torrance, CA, USA) and then stored in the freezer at -80°C until examined. The frozen brains were subsequently cut at a thickness of 10 μ m using a Leica CM 3050 Cryostat (Leica Microsystems, Wetzlar, Germany), after which the sections were immunostained with antibodies against A β (Covance, Emeryville, CA, USA), p-tau (Sigma-Aldrich) and CD11b (Serotec, Oxford, UK). Following additional incubation with biotinylated secondary antibody, samples were incubated in ABC reagent (Vector Laboratories, Burlingame, CA, USA). All reactions were visualized by development in 3,3'-diaminobenzidine substrates (Vector

Laboratories), and all samples were visualized using a light microscope (Carl Zeiss, Jena, Germany). The immunoreaction products were quantified using the iSolution full image analysis software (Image & Microscope Technology, Vancouver, BC, Canada).

Morris water maze task. Spatial learning and memory deficits were assessed using the Morris water maze task as previously described (11) with minor modification. The experiment was performed on mice after a 1- or 4-week A β_{25-35} injection period. The maze consisted of a 1.15-m-diameter pool that was painted flat white and had a 10-cm-diameter platform halfway between the center of the pool and the edge, 1 cm below the surface of the water. The water in the pool was made opaque by the addition of powdered milk. The water temperature was 19–21°C. The water tank was located in a test room that contained several cues external to the maze. The position of the cues remained unchanged throughout the water-maze task. Each mouse was subjected to a series of 5 trials/day. For each trial, mice were randomized to 1 of 4 directional starting locations (north, south, east and west) and placed in the pool facing the wall. During the 4 subsequent training days, the mice were given 5 trial sessions/day with the platform in place before an A β_{25-35} injection. When a mouse located the platform, it was permitted to remain on it for 10 sec. If the mouse did not locate the platform within 180 sec, it was placed on the platform for 10 sec. Mice were given a maximum of 180 sec to find the submerged platform. Swimming was videotaped, and the escape latency time to reach the platform was analyzed using the Smart software (Panlab, Barcelona, Spain).

Data analysis. All data are expressed as the means \pm SEM. Statistical comparisons were performed using paired or unpaired Student's t-tests, and one-way analysis of variance (ANOVA) or two-way ANOVA for repeated measures followed by Fisher's protected least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hypercholesterolemia increases A β , p-tau and CD11b expression in A β -injected mice. Plasma cholesterol levels were significantly higher in Apo E KO mice that were fed a high-fat diet than in control mice fed normal chow (737.56 \pm 27.58 vs. 99.4 \pm 7.58 mg/dl, respectively, $P < 0.01$), indicating that administration of the high-fat diet for 8 weeks induced hypercholesterolemia. To validate the function of hypercholesterolemia on AD *in vivo*, A β_{25-35} was used for the AD-like pathological model in this study. Unilateral icv injection of A β_{25-35} led to increased immunoreactivity for the AD-related neuropathological markers A β and p-tau in the hippocampus (Figs. 1 and 2). Moreover, hypercholesterolemia tended to increase A β and p-tau levels in the vehicle group and further increased A β and p-tau levels in A β_{25-35} -injected mice. CD11b (also known as $\alpha_m\beta_2$ integrin and Mac1) is a sensitive marker of microglial activation. Immunostaining of CD11b was evident in the hippocampus of normal mice receiving icv injections of A β_{25-35} (Fig. 3). Consistent with

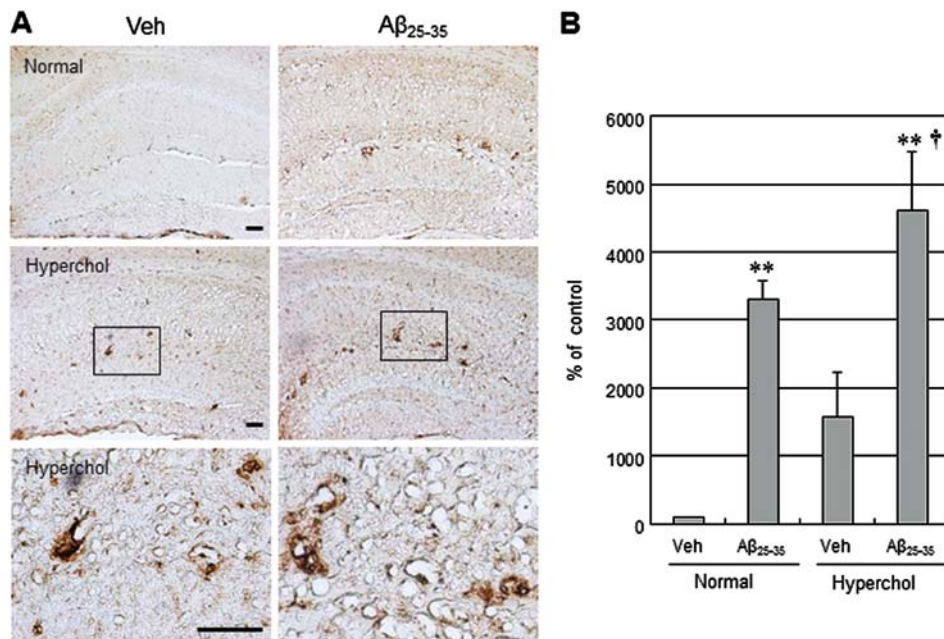


Figure 1. Hypercholesterolemia increases Aβ levels in the Aβ₂₅₋₃₅-injected mouse brain. Coronal sections of the Aβ₂₅₋₃₅-injected mouse brain were stained immunohistochemically with Aβ antibody. Hypercholesterolemia in Apo E KO mice was induced by providing a high-fat diet for 4 weeks prior to Aβ₂₅₋₃₅ injection and for 4 weeks following Aβ₂₅₋₃₅ injection, for a total of 8 weeks. (A) The rectangle represents the imaging field. (B) Quantification of Aβ-positive cells is expressed as the % change of the vehicle group of normal mice. Unilateral icv injection of Aβ₂₅₋₃₅ yielded a significant increase in immunoreactivity for Aβ in both normal and hypercholesterolemic mice (Hyperchol) and hypercholesterolemia led to increased Aβ levels when compared to normal mice. **P<0.01 vs. vehicle group of normal mice; †P<0.05 vs. vehicle group of hypercholesterolemic mice. Values are the means ± SEM. N=4. Scale bar, 50 μm.

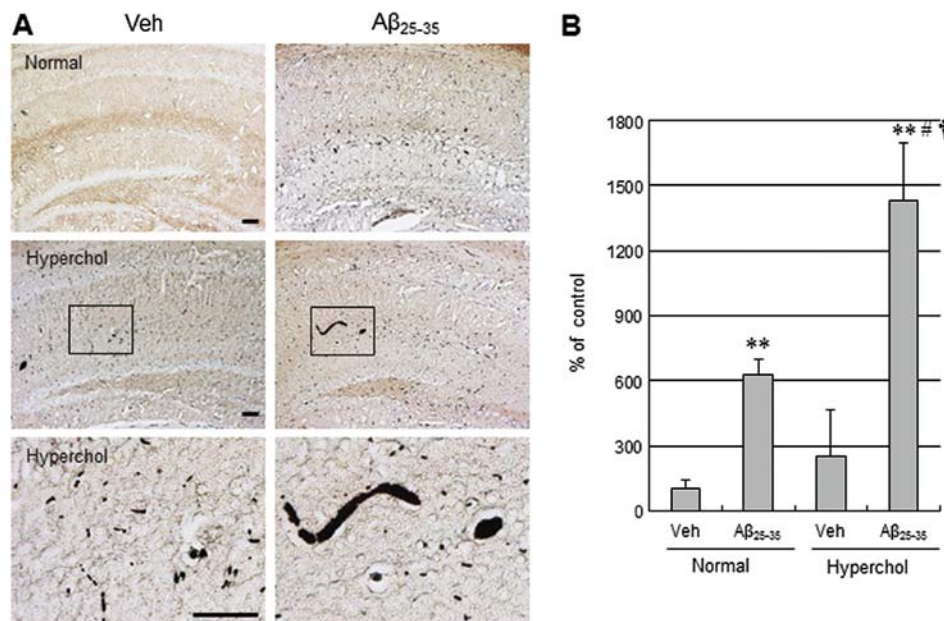


Figure 2. Hypercholesterolemia increases tau phosphorylation in the Aβ₂₅₋₃₅-injected mouse brain. Coronal sections of the Aβ₂₅₋₃₅-injected mouse brain were stained immunohistochemically with p-tau antibody. Hypercholesterolemia in Apo E KO mice was induced by providing a high-fat diet for 4 weeks prior to Aβ₂₅₋₃₅ injection and for 4 weeks following Aβ₂₅₋₃₅ injection, for a total of 8 weeks. (A) The rectangle represents the imaging field. (B) Quantification of p-tau-positive cells is expressed as the % change of the vehicle group of normal mice. Unilateral icv injection of Aβ₂₅₋₃₅ significantly increased immunoreactivity for p-tau in both normal and hypercholesterolemic mice (Hyperchol) and hypercholesterolemia significantly led to increased p-tau levels when compared to normal mice. **P<0.01 vs. vehicle group of normal mice; †P<0.05 vs. Aβ₂₅₋₃₅-injected group of normal mice; ‡P<0.05 vs. vehicle group of hypercholesterolemic mice. Values are the means ± SEM. N=4. Scale bar, 50 μm.

Aβ and p-tau, hypercholesterolemia led to a more significant increase in CD11b immunostaining in both the vehicle group and the Aβ₂₅₋₃₅-injected group of hypercholesterolemic mice.

However, there were no significant changes in GFAP immunoreactivity in normal and hypercholesterolemic mice treated following Aβ₂₅₋₃₅ icv injections (data not shown).

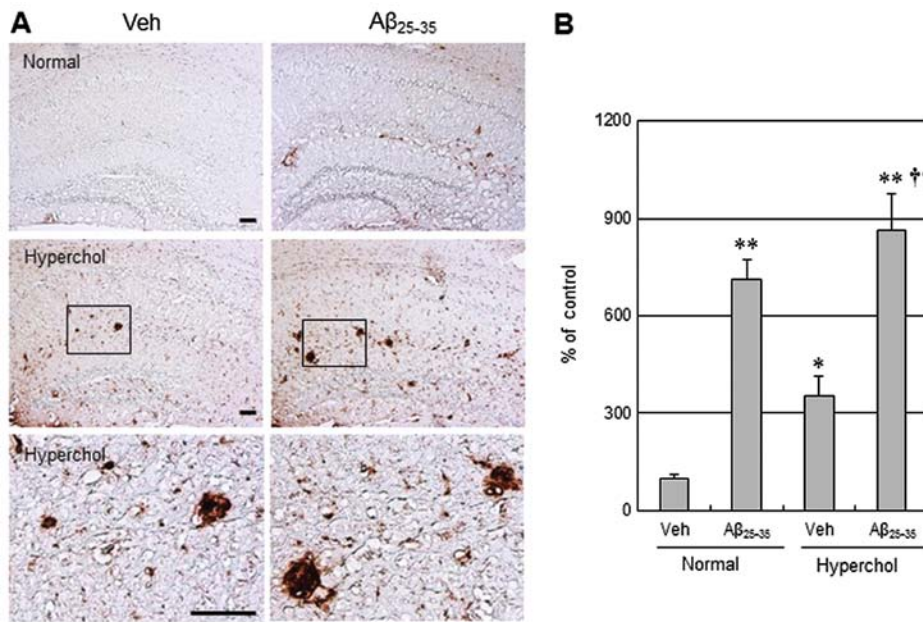


Figure 3. Hypercholesterolemia increases CD11b in the A β_{25-35} -injected mouse brain. Coronal sections of the A β_{25-35} -injected mouse brain were stained immunohistochemically with CD11b antibody. Hypercholesterolemia in Apo E KO mice was induced by providing a high-fat diet for 4 weeks prior to A β_{25-35} injection and for 4 weeks following A β_{25-35} injection, for a total of 8 weeks. (A) The rectangle represents the imaging field. (B) Quantification of CD11b-positive cells is expressed as the % change of the vehicle group of normal mice. Unilateral icv injection of A β_{25-35} yielded a significant increase in immunoreactivity for CD11b in both normal and hypercholesterolemic mice (Hyperchol) and hypercholesterolemia led to increased CD11b levels when compared to normal mice. * $P < 0.05$; ** $P < 0.01$ vs. vehicle group of normal mice; †† $P < 0.01$ vs. vehicle group of hypercholesterolemic mice. Values are the means \pm SEM. $N = 4$. Scale bar, 50 μ m.

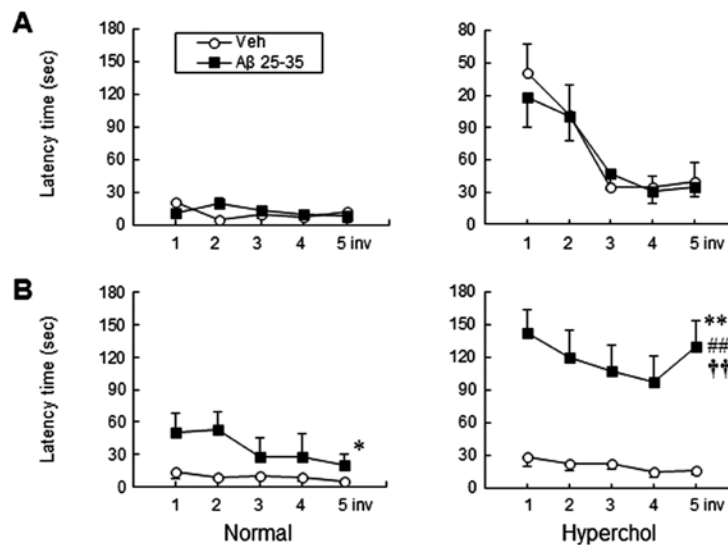


Figure 4. Effects of hypercholesterolemia on spatial learning and memory impairment in A β_{25-35} -injected mice as determined by the Morris water maze test. The changes in escape latency to reach the platform in normal mice and hypercholesterolemic mice (Hyperchol) at (A) 1 week and (B) 4 weeks following A β_{25-35} injection. A β_{25-35} significantly increased escape latency time at 4 weeks after A β_{25-35} injection and hypercholesterolemia led to increased latency time when compared to normal mice. * $P < 0.05$; ** $P < 0.01$ vs. vehicle group of normal mice; †† $P < 0.01$ vs. A β_{25-35} -injected group of normal mice; †† $P < 0.01$ vs. vehicle group of hypercholesterolemic mice. Values are the means \pm SEM. $N = 7$.

Hypercholesterolemia aggravates cognitive impairment in A β -injected mice. To verify whether hypercholesterolemia has any effect on cognitive deficits, we subjected the mice to a water maze. As shown in Fig. 4, icv injection of A β_{25-35} resulted in a significantly increased escape latency time in the target quadrant when compared to the vehicle group at 4 weeks after A β injection ($P < 0.05$ vs. vehicle) (Fig. 4B). Conversely, no significant difference was observed among

groups at 1 week after A β injection (Fig. 4A). Moreover, hypercholesterolemia significantly exacerbated the cognitive decline when compared to the A β_{25-35} -injected group of normal mice at 4 weeks after A β injection ($P < 0.01$, two-way ANOVA for repeated measures). Thus, decreased spatial learning in response to hypercholesterolemia was coincident with accelerated expression of A β and p-tau and activation of microglia in the A β_{25-35} -injected group of hypercholesterolemic mice.

Discussion

In the present study, we examined the effects of hypercholesterolemia on the cognitive deficits in A β ₂₅₋₃₅-injected mice with respect to neuroinflammatory and AD-related biochemical changes. Our results demonstrated that hypercholesterolemia strongly accelerated A β accumulation, p-tau expression and microglial activation in the brains of A β ₂₅₋₃₅-injected mice. In addition, hypercholesterolemia significantly aggravated spatial learning and memory in A β ₂₅₋₃₅-injected mice as assessed by the Morris water maze test. These data suggest that hypercholesterolemia accelerates the AD-related pathology, which was accompanied by neuroinflammation and subsequent memory impairment in this mouse model. Thus, we propose that diet can be used to modulate the risk of developing AD.

Clinical studies indicate that patients with elevated cholesterol have increased susceptibility to AD (12-14) and that lowering plasma cholesterol with statin therapy during middle age is likely to benefit cognitive function in later life (15,16). Experimental studies using rabbits (3) and a transgenic mouse model of AD (4-6) have demonstrated that diet-induced hypercholesterolemia increased A β levels in the brain and thus accelerated extracellular A β deposition. A β depositions and neurofibrillary tangles are distinctive hallmarks of AD. A disturbed cholesterol homeostasis within lipid rafts might influence amyloid precursor protein (APP) processing, which results in increased A β production and deposition (17,18). Conversely, little is known about the function of cholesterol in the formation of neurofibrillary tangles. Our data revealed that hypercholesterolemia enhanced A β as well as p-tau levels in the brain, which was confirmed by descriptive immunohistochemical analysis. Our findings are in accordance with previous studies showing elevated concentrations of A β (4,19,20) and an enhanced level of hyperphosphorylated tau (20) after consumption of a cholesterol-enhanced diet.

AD is characterized by cerebrovascular damage and neuronal dysfunction, which leads to progressive cognitive decline. Despite clear evidence of hypercholesterolemia-induced amyloid pathology, the relationship between hypercholesterolemia and memory impairment in animals is somewhat controversial (21-23). In this study, hypercholesterolemia led to significant exacerbation of cognitive decline when compared to the A β ₂₅₋₃₅-injected group of normal mice at 4 weeks after A β injection. Similar to our findings, aggravation of learning deficits in association with A β deposition in APP transgenic mice with low density lipoprotein receptor (LDLR)-deficiency has been reported (24).

The exact mechanisms responsible for the cognitive deficit induced by hypercholesterolemia in mice remain unknown. One possible explanation is that a oxidative-inflammatory cycle in the vasculature could have deleterious consequences for brain function and cognition (25,26). It is well known that inflammation occurs in AD brains and hypercholesterolemic animal models (8,26). Our data demonstrate that hypercholesterolemia induced elevated immunohistochemical-positive staining for the microglial marker CD11b. There is now convincing evidence that inflammatory cascades primarily mediated by activated microglia play a significant

role in AD-like changes (9,27). The mechanism of microglial activation and the role of hypercholesterolemia in the process of neuroinflammation are not fully known. It is possible that the systemic inflammation under hypercholesterolemic conditions, which causes dysfunctional cerebrovasculature, may trigger activation of the perivascular microglia (28) and recruitment of microglia to the inflamed brain arterioles could then initiate a cascade of events leading to AD-like pathology and cognitive dysfunction.

The effects of hypercholesterolemia *in vivo* have been studied in different animal models, and experimental studies have shown that hypercholesterolemia increases brain A β deposition and neuroinflammation in rabbits (3), soluble APP transgenic mice (4) and LDLR deficit mice (26). In the present study, we used Apo E KO mice on a high-fat diet to induce hypercholesterolemia and A β ₂₅₋₃₅, a toxic core fragment of full-length A β ₁₋₄₀ (29), to model AD-like pathology. Although it remains unclear whether the A β ₂₅₋₃₅ fragment occurs in AD brains, a number of studies have demonstrated that the acute injection of this peptide into the cerebral ventricle results in some pathological events observed in AD, such as neurotoxic effects similar to those produced by A β ₁₋₄₀ (30) or impairment of memory in mice (31). To the best of our knowledge, the present study is the first to examine the hypercholesterolemic effects in an A β ₂₅₋₃₅-injected AD-like pathological mouse model.

In conclusion, the present study supports the findings of clinical studies suggesting that a high-fat diet increases the risk of developing AD and therefore suggests that diet could be used to reduce the risk of developing the disease. These findings suggest that dietary methods might offer a path to the prevention of AD without resorting to drugs that are expensive and may have harmful side-effects.

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