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CHANGES IN ASTROCYTE FUNCTIONAL MARKERS AND β-AMYLOID METABOLISM-RELATED PROTEINS IN THE EARLY STAGES OF HYPERCHOLESTEROLEMIA

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Abstract—Cholesterol is an essential substance for maintaining normal structure and function of the brain. But unfortunately, a long-term high-cholesterol diet can lead to a variety of pathological changes of the brain such as β-amyloid (Aβ) accumulation, Tau hyperphosphorylation, reactive gliosis, neuroinflammation, neuronal death and synaptic degeneration. These pathological changes have complex internal relations with one other, causing memory impairment and participating in the pathogenesis of Alzheimer's disease (AD). However, early hypercholesterolemia-induced events that lead to brain deterioration are not clear. To address this, 6-month-old female mice were fed a 3% cholesterol diet for 8 weeks, followed by behavioral, biochemical and neuropathological analyses. The high-cholesterol-fed mice did not show neuronal and synaptic impairment or cognitive deficits compared with mice given a normal diet, but astrocytes were mildly activated with increased expression of functional markers including apolipoprotein E and aquaporin 4 in the hippocampus. Hippocampal interleukin-1β expression slightly increased, but interleukin-6 (IL-6) and tumor necrosis factor-α did not change significantly compared with those in the control group. Levels of Aβ, and its precursor protein, were unaffected, but levels of presenilin 1 and insulin-degrading enzyme (IDE), that initiate Aβ generation and degradation, respectively, increased in the hippocampus of the model mice. In addition, Tau phosphorylation levels were not different between the control and model groups. These results suggest that changes in astrocyte functional markers and Aβ metabolism proteins, which contribute to maintaining brain cholesterol and Aβ homeostasis, are early events in the process of hypercholesterolemia-related neuropathological changes.

Key words: hypercholesterolemia, astrocytes, Alzheimer's disease, β-amyloid, neuroinflammation.

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease, and is predicted to increase in incidence in the coming years. The pathogenesis of AD is not entirely understood, and is thought to be associated with various factors including age, heredity, environment, traumatic brain injury, lifestyle and diet habits (Holtzman et al. 2011). Exploring the underlying mechanisms of the risk factors contributing to AD development will be helpful in establishing new strategies for the prevention and treatment of this devastating disease.

Cholesterol is an essential nutrient and an important component of cell membrane and plasma lipoprotein. The brain contains the highest levels of cholesterol in the body, and it is vital in brain functions including signal transduction, neurite growth, synaptic plasticity, learning and memory (Dietschy and Turley, 2001; Schreurs, 2010). It is commonly viewed that cholesterol cannot freely cross the blood–brain barrier (BBB) in physiological condition, and brain cholesterol metabolism originates from *in situ* neo-synthesis (Zhang and Liu, 2015). Cholesterol in the adult brain is synthesized primarily by astrocytes, bound to apolipoprotein E (APOE), and taken up by neurons via the low-density lipoprotein receptor (Quan et al., 2003). However, a high-cholesterol diet not only increases plasma cholesterol levels, but also affects brain cholesterol homeostasis, causing a series of pathological changes associated with the occurrence and development of AD (Howland et al., 1998; Refolo et al., 2000; Liu et al., 2010a).

A large amount of evidence suggests a pathogenic link between hypercholesterolemia and AD, although the exact mechanisms are still unclear (Simons et al., 1998; Puglielli et al., 2003; Ricciarelli et al., 2012). Animals fed

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Abbreviations: AD, Alzheimer's disease; ADAM10, a-disintegrin and metalloproteinase 10; APOE, apolipoprotein E; APP, amyloid precursor protein; AQP4, aquaporin-4; Aβ, β-amyloid; BACE1, β-site amyloid precursor protein-cleaving enzyme 1; BBB, blood–brain barrier; GFAP, glial fibrillary acidic protein; GLT1, glutamate transporter 1; Iba-1, ionized calcium-binding adaptor molecule 1; IDE, insulin-degrading enzyme; IL-1β, interleukin-1β; IL-6, interleukin-6; LRP1, low-density lipoprotein receptor-related protein 1; NEP, neprilysin; PCR, polymerase chain reaction; PS1, presenilin1; PSD95, postsynaptic marker postsynaptic density protein 95; sAPPα, soluble amyloid precursor protein α; SYP, synaptophysin; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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a high-cholesterol diet show increases in β-amyloid (Aβ) generation and accumulation (Sparks et al., 1994; Ghribi et al., 2006; Thirumangalakudi et al., 2008; Jaya Prasanthi et al., 2008; Umeda et al., 2012). Abnormal brain cholesterol metabolism can increase Tau protein expression and phosphorylation (Ghribi et al., 2006). More importantly, the long-term experimental hypercholesterolemia causes hippocampal neuroinflammatory and neurodegeneration with corresponding cognitive deficits (Sparks et al., 2000;Thirumangalakudi et al., 2008; Lu et al., 2010; Umeda et al., 2012; Moreira et al., 2014). Consistently, epidemiological investigations also show that elderly patients with hypercholesterolemia had an increased incidence of mild cognitive impairment, or even AD, when compared with the normal population (Pappolla et al., 2003; Solomon et al., 2009). Furthermore, the aforementioned neuropathological changes caused by hypercholesterolemia are not autonomous, but influence each other (Liu et al., 2010a). Amyloid plaque deposition evokes the sustained activation of adjacent microglia and astrocytes, subsequently causing the release of inflammatory factors (Tanzi and Bertram, 2005). As a result, the inflammatory reaction is intensified, which in turn promotes Aβ accumulation, oxidative damage and neurodegeneration (Niranjan, 2013). Thus, these complex pathological events have a close interrelationship, and participate simultaneously in the onset and pathogenesis of AD. However, which early events are attributed to the hypercholesterolemia-related neuropathology is not clear. Therefore, identifying the initial events will help to block the cascade interaction mentioned above, and prevent the occurrence and development of AD caused by hypercholesterolemia.

In a previous study, we demonstrated that adult female C57BL/6 mice receiving a normal diet, plus 3% cholesterol for 9 weeks, have increased plasma cholesterol levels, but do not produce cognitive impairment associated with hippocampal neuronal and synapse loss (Li et al., 2012). By use of this hypercholesterolemic model, the present study was designed to determine the extent of changes in Aβ metabolism and accumulation, glial activation, inflammatory reaction, and phosphorylation levels of Tau protein in the hippocampus. Our results suggest that mild astrocyte activation, accompanied by their functional plasticity changes, including cholesterol transport and inflammatory factor secretion, occurs prior to neuronal degeneration caused by hypercholesterolemia. In addition, changes in expression levels of Aβ production and clearance-related enzymes may contribute to maintaining Aβ homeostasis during the early stages of hypercholesterolemia.

EXPERIMENTAL PROCEDURES

Animals and experimental design

Six-month-old female C57/BL6 mice were housed 4 per cage in a room of controlled illumination (12:12 h light/dark cycle), humidity (30–50%), and temperature (18–22 °C). Food and water were available *ad libitum*. After 1-week of acclimatization, the mice were randomly divided into two groups (n = 10 in each group): control diet and high-cholesterol diet (HC). They were fed either a normal diet, or one containing 3% cholesterol (Sigma–Aldrich; Cat. No: C8503) for 8 weeks. The detailed information about diet components is available in Table 1. Body weight was measured once a week. All experiments were conducted in accordance with international standards on animal welfare and the guidelines of the Institute for Laboratory Animal Research of Nanjing Medical University. All efforts were made to minimize animal suffering and reduce the number of animals used.

Y-maze test

The Y maze test was performed to measure mice short-term memory, as previously described (Wang et al., 2013). The Y maze included 3 arms: novel arm (NA), starting arm (SA), and other arm (OA). The test contains two stages: training stage and testing stage. During the first stage, the NA was blocked by a black baffle. The mice were allowed to move freely only between the SA and OA; during the second stage, the NA was opened and mice could freely move throughout all 3 arms. The percentage of time spent in each arm and the number of entries into each arm were calculated.

Blood and brain tissue sample preparation

The mice were deeply anesthetized with 3.5% chloral hydrate (1 ml/100 g, i.p.). Body weight was measured and blood was collected from the orbital vein. For cholesterol analysis, blood samples were centrifuged at 3000 rpm for 30 min at 4 °C in a microcentrifuge (Thermo Scientific MicroCL 17R, 1.5/2 ml rotors) to separate serum, then stored at −20 °C until ready for assay. For Western blotting, mice were decapitated and the brains rapidly removed. The hippocampus was dissected bilaterally and stored at −80 °C. For pathological analysis, mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were removed, post-fixed overnight at 4 °C, dehydrated in a series of graded ethanol solutions and embedded in paraffin. The coronal sections were serially

Table 1. Diet composition

Ingredient	Control diet (%)	Cholesterol diet (%)
Corn starch	43	43
Wheat bran	34	34
Soybean flour	12	9
Bonemeal	5	5
Soybean oil	2	2
Cod liver oil	1	1
Salt	1	1
Mineral mix	1.25	1.25
Vitamin mix	0.75	0.75
Cholesterol	0	3

Diets were made by Nanjing AnLiMo technology development co., LTD. The soybean flour (12%) in the control diet was replaced with 3% cholesterol + 9% soybean flour in the cholesterol diet. Water-soluble cholesterol (3β-hydroxy-5-cholestene, 386.65 g/mol) was purchased from Sigma–Aldrich (Product Number: C8503). Its biological source is from sheep wool with ⩾92.5% (GC) purity and powder appearance.