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β-sitosterol inhibits high cholesterol-induced platelet β-amyloid release

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Abstract Recently, increasing evidence has linked high cholesterol to the pathogenesis of Alzheimer's disease (AD), suggesting that cholesterol may be a target for developing new compounds to prevent or treat AD. Plant sterols, a group of sterols enriched in plant oils, nuts, and avocados, have the structure very similar to that of cholesterol, and have been widely used to reduce blood cholesterol. Due to their cholesterol-lowering property, plant sterols such as β-sitosterol may also influence cholesterol-depending functions including its role in AD development. Using human platelets, a type of peripheral blood cells containing the most circulating amyloid precursor protein (APP), this study investigated the effect of β-sitosterol on high cholesterol-induced secretion of β amyloid protein (Aβ). It was found that β-sitosterol effectively inhibited high cholesterol-driven platelet Aβ release. In addition, β-sitosterol prevented high cholesterol-induced increase of activities of β- and γ-secretase, two APP cleaving enzymes to generate Aβ. Additional experiments showed that high cholesterol up-regulated lipid raft cholesterol. This effect of cholesterol could be suppressed by β-sitosterol. These findings suggest that β-sitosterol is able to inhibit high cholesterol-induced Aβ release probably through maintenance of membrane cholesterol homeostasis. Given that dietary plant sterols have the potential of penetrating the blood–brain barrier (BBB), these data suggest that plant sterols such as β-sitosterol may be useful in AD prevention.

Keywords Cholesterol · Plant sterols · β-sitosterol · β amyloid protein (Aβ) · Platelets

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Introduction

AD is a progressive neurodegenerative disease characterized by the deposition of amyloid-β (Aβ) in senile plaques, the appearance of neurofibrillary tangles, neuronal loss, synaptic damage, and cholinergic deficits (Shi et al. 2008a). The overproduction and accumulation of Aβ peptides in the brain have been considered key factors underlying the pathogenesis of AD (Stefani and Liguri 2009). Aβ is derived from the amyloid precursor protein (APP) by proteolytic processing (Gandy 1999). Normally, APP is processed by two alternative pathways, one that involves cleavage by α-secretase and then by γ-secretase, and another that involves cleavage by β-secretase and then by γ-secretase (Goodenough et al. 2003). The former pathway results in the secretion of sAPPα

and p3, which are both soluble and nontoxic; the latter pathway results in the secretion of soluble sAPPβ and insoluble amyloidogenic Aβ (Goodenough et al. 2003). The factors that influence the switch between the two APP processing pathways remain to be determined.

Recently, increasing evidence has linked cholesterol to APP processing (Wolozin 2001; Miller and Chacko 2004; Puglielli et al. 2005; Xiong et al. 2008). ApoE4 allele (The epsilon4 allele of the apolipoprotein E gene) carriers are predisposed toward an earlier onset of AD (Corder et al. 1993; Runz et al. 2002). A high cholesterol diet accelerates Aβ generation in APP transgenic mice (Refolo et al. 2000; Runz et al. 2002) while suppression of cholesterol biosynthesis strongly reduces the production of Aβ species in vivo and in vitro (Simons et al. 1998; Frears et al. 1999; Refolo et al. 2000; Fassbender et al. 2001; Runz et al. 2002). Cholesterol is an essential membrane component of mammalian cells and is highly concentrated in specialized membrane microdomains termed lipid rafts. Cholesterol-rich lipid raft is closely associated with APP processing (Bouillot et al. 1996; Riddell et al. 2001; Runz et al. 2002; Beel et al. 2010). It is known that two pools of APP are distributed inside and outside lipid rafts, respectively (Xiong et al. 2008). Since β- and γ-secretase are localized to cholesterol-rich lipid rafts (Bouillot et al. 1996; Riddell et al. 2001; Runz et al. 2002), APP usually undergoes β- and γ-secretase cleavage within lipid rafts. In addition, recent evidence suggests that membrane cholesterol favors β-secretase cleavage of APP by direct binding to the C-terminal transmembrane domain (C99) of APP (Beel et al. 2010). Therefore, membrane cholesterol may be one of the factors that determine APP processing pathways and Aβ production in AD brains.

Plant sterol (phytosterol) is a group of sterols enriched in plant oils, nuts, and avocados, with the structure very similar to that of cholesterol (Jansen et al. 2006). They differ from cholesterol only by an additional side chain at the C24- or C22-position (Jansen et al. 2006). But this small structural difference may lead to differential effects of plant sterols and cholesterol in mammalian cells. Recently, food products containing plant sterols have been widely used as a therapeutic diet to lower plasma cholesterol and atherosclerotic risk (Calpe-Berdiel et al. 2009). A high plant sterol intake (2–2.5 g/day) was demonstrated to decrease levels of serum cholesterol significantly (Pollak and Kritchevsky 1981; Clifton 2009). However, the cholesterol-lowering mechanism of plant sterols is not yet fully known. It has been suggested that plant sterols inhibit intestinal cholesterol absorption, possibly by competing with cholesterol to incorporate into the mixed micelles, by replacing cholesterol in bile, or by preventing cholesterol esters from hydrolysis in the small intestine (Ikeda et al. 1989; Ling and Jones 1995; Ostlund 2002; Richelle et al. 2004). In addition, plant sterols and their derivatives are suggested to be ligands for liver X receptor (LXRα and LXRβ) (Kaneko et al. 2003; Plat et al. 2005; Kim et al. 2008). Through activation of LXRα, plant sterols may trigger cholesterol efflux (Kim et al. 2008). Due to the cholesterol-lowering property, plant sterol can be expected to influence cholesterol-dependent functions, including the role of cholesterol in AD development. To test this possibility, in this study, we investigated whether the β-sitosterol, one of the most common forms of plant sterols, influenced APP metabolism in the presence of high levels of cholesterol.

Platelets, a readily accessible source of living human tissue, have long been used as a model to study APP metabolism (Evin et al. 2003). The rationale of using platelets is that platelets contain more than 95% of the circulating APP, and also contain all the enzymatic machinery (α-, β- and γ-secretase) to generate the by-products of APP cleavage (Li et al. 1998; Zainaghi et al. 2007). Platelets of AD patients display increased levels of Aβ, increased expression of BACE1 (a β-secretase), and decreased expression of ADAM10 (an α-secretase), supporting their usefulness in studying AD pathogenetic mechanisms (Zainaghi et al. 2007; Shi et al. 2008b). Accordingly, this study use human platelets to test the effect of β-sitosterol on high cholesterol-driven Aβ release.

Methods

Reagents

D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Unless stated otherwise, all other chemicals were from Sigma (St. Louis, MO, USA). Stock solutions (2000X) of cholesterol, β-sitosterol, prostaglandin E1 and PPACK were prepared in ethanol and added to the platelet suspension at the indicated concentrations. Stock solutions of collagen (5 mg/ml) were prepared in water (pH was adjusted to 3.0 with acetic acid). In all experiments, an equivalent volume of ethanol (0.05%) was added to the control group.

Subjects

Twelve healthy control subjects (6 men and 6 women, mean age 30.7±5.19 years, range 23–40 years, education 20±1.75 years) were recruited, all without complaints of cognitive or memory deficits. Major systemic, psychiatric, neurological illness, hypertension, diabetes mellitus, tumor, drug or alcohol abuse, intake of anticoagulants, autoimmunologic diseases and hypercholesterolaemia were pre-

cluded for all subjects. Plasma total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein cholesterol for all the subjects were 4.3±0.3, 2.2±0.2, 2.0±0.2, respectively. Written informed consent from all subjects was obtained before the study commenced.

Platelet-rich plasma (PRP) preparation and pharmacological treatments

Ten milliliter peripheral venous blood was collected from each subject. The blood was immediately centrifuged at 150 g for 20 min at room temperature (Gadd and Clayman 1972). The supernatant was the PRP (Gadd and Clayman 1972). The platelet count of all PRP was adjusted to 1×10^8/ml by platelet-free plasma obtained by centrifugation of the blood samples at 4,000 g for 20 min.

To determine the effect of β-sitosterol or cholesterol on platelet APP metabolism, 1–3 mM of cholesterol or 5–15 μM of β-sitosterol was added to PRP. The PRP was then incubated for 24 h. During the incubation, platelet suspension was kept stagnant in a cell culture incubator at 37 °C. To determine whether β-sitosterol influences the effect of high cholesterol on platelet Aβ release, platelets were pre-incubated with β-sitosterol (15 μM) at 37 °C for 2 h followed by simultaneous challenge with cholesterol (2 mM) and β-sitosterol (15 μM) for another 24 h.

For measurement of Aβ in the media, after cholesterol and/or β-sitosterol treatment, platelets were collected by centrifugation at 1,400 g for 15 min at room temperature and re-suspended in modified Tyrode buffer (150 mM NaCl, 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.55 mM NaH_2PO_4, 7 mM $NaHCO_3$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose,40 μg/ml PPACK) (Gadd and Clayman 1972; Chang et al. 1998). Platelet Aβ release in the medium was examined at rest and in the presence of collagen (Human placenta collagen type I, 10 μg/ml). To make platelets at rest, 1 μM prostaglandin E1 was added to platelet suspension to prevent platelet activation by any agitation (e.g., centrifugation) (Connolly et al. 1992; Roberts et al. 2010). For measurement of Aβ released from resting and activated platelets, platelet suspension was pre-incubated at 37 °C for 5 min. Then, saline or collagen (10 μg/ml final concentration) was added and incubation continued for 10 min. After incubation, the platelet suspension was centrifuged (4,000 g for 20 min at 4 °C), and a 100-μl sample of the platelet-free supernatant was used for measurement of platelet secretion of Aβ.

Alternatively, after cholesterol and/or β-sitosterol treatments, platelet pellets were collected by centrifugation of the PRP at 1,400 g for 15 min and then used for measurement of the activities of β- and γ-secretase as well as levels of lipid raft cholesterol.

Aβ measurement

Platelet secretion of Aβ was measured by chemiluminescence using a human Aβ ELISA kit (#SIG-38966, BetaMark, Covance, Dedham, MA) according to the manufacturer's protocol. The chemiluminescent data were collected using the Victor-2 Multilabel counter (Perkin Elmer/Wallace).

β- and γ-secretase activities

β-secretase activity in platelets was measured by fluorescent spectroscopy using a β-secretase activity detection kit (#CS0010, Sigma, Saint Louis, USA) according to the manufacturer's protocol. Briefly, platelets were lysed in CelLytic™-M and centrifuged for 10 min at 14,000 g. Then 20 μl of the supernatant was used for β-secretase activity measurement. The cleavage-dependent fluorescence signal was quantified using the same Victor-2 Multilabel counter at an excitation wavelength of 320 nm and emission wavelength of 405 nm.

γ-secretase activity in platelets was measured by fluorescent spectroscopy using a γ-secretase activity kit according to the manufacturer's instructions (#FP003, R&D Systems, Minneapolis, MN,. USA). The cleavage-dependent release of the fluorescence signal was quantified using the same Victor-2 Multilabel counter at an excitation wavelength of 340 nm and emission wavelength of 495 nm.

The protein concentration of platelet pellets was determined with a Lowry-based DC (Detergent Compatible) protein assay kit (Bio-Rad, Hercules, CA, USA). Spectrophotometric data were obtained using the same Victor-2 Multilabel counter at a wavelength of 650 nm. Enzyme activities were corrected for protein content.

Levels of lipid raft cholesterol

Lipid rafts were isolated from platelet membrane by discontinuous sucrose density gradient as described previously (Clement et al. 2010). Briefly, platelet pellets were re-suspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 mM CHAPS). After incubation on ice for 30 min, the lysate was mixed with 40% sucrose solution. The 40% sucrose solution was then overlaid by a step gradient of 30% sucrose and 5% sucrose (v/v/v: 12:26:5). After centrifugation for 120 min at 200,000 g at 4 °C, fragments floating at the interface between 5 and 30% sucrose were collected and a 50-μl sample of fragments was used for cholesterol measurement.

Free unesterified cholesterol levels were analyzed by fluorescent spectroscopy using the Amplex red cholesterol assay kit (Invitrogen, UK) according to the manufacturer's

protocol. The fluorescent data were collected using the same Victor-2 Multilabel counter at an excitation wavelength of 560 nm and emission wavelength of 590 nm.

Statistical analysis

Statistical analysis was done by SPSS 15.0 software (Statistical Package for the Social Sciences). After the Kolmogorov–Smirnov test for normal distribution and Levene's test of Equal Variances for variance homogeneity, data were analyzed by one-way analysis of variance (ANOVA) followed by pairwise t tests.

Results

β-sitosterol inhibits high cholesterol-induced platelet Aβ release

To test the effects of cholesterol or β-sitosterol on platelet Aβ release, platelets were incubated with different concentrations of cholesterol or β-sitosterol for 24 h. It was found that cholesterol concentration-dependently increased Aβ released from resting platelets ($P<0.01$, Fig. 1a). Cholesterol also enhanced collagen-triggered platelet-soluble Aβ release in a concentration-dependent manner ($P<0.01$, Fig. 1a). By contrast, incubation of platelets with different concentrations of β-sitosterol for 24 h did not affect Aβ efflux from resting platelets or collagen-activated platelets ($P>0.05$, Data not shown).

To investigate whether β-sitosterol affected high cholesterol-induced platelet Aβ release, we added cholesterol (2 mM) and β-sitosterol (15 μM) into the PRP. We found that β-sitosterol at 15 μM abrogated high cholesterol-induced Aβ release whether from the resting platelets or from the collagen-activated platelets ($P<0.01$, Fig. 1b).

β-sitosterol inhibits high cholesterol-induced increased activities of β- and γ-secretase

We have shown the effects of cholesterol and/or β-sitosterol on platelet Aβ release. Next, we tested the effects of cholesterol and/or β-sitosterol on the activities of β- and γ-secretase, two enzymes that generate Aβ. We found that high cholesterol increased the activities of platelet β- and γ-secretase ($P<0.01$, Figs. 2 and 3). By contrast, exposure of platelets to β-sitosterol (15 μM) for 24 h did not affect β- or γ-secretase activity ($P>0.05$, data not shown).

To determine whether β-sitosterol influenced high cholesterol-induced increase of β- and γ-secretase activities, we incubated platelets with cholesterol (2 mM) and β-sitosterol (15 μM). We found that β-sitosterol at 15 μM

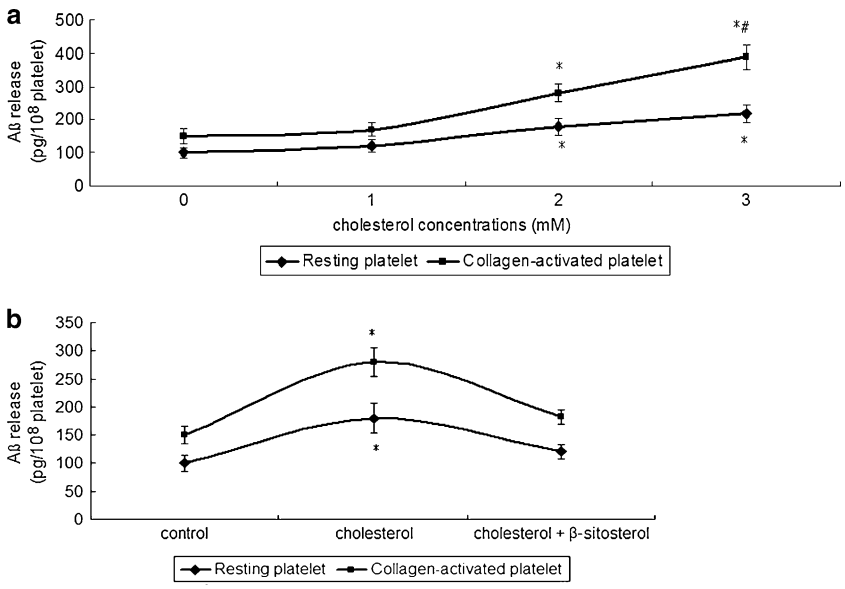

Fig. 1 β-sitosterol inhibits high cholesterol-induced platelet Aβ release. **a** Different concentrations of cholesterol were added to PRP. The PRP was then incubated at 37 °C for 24 h. After that platelets were collected and re-suspended in modified Tyrode buffer. Platelet Aβ release in the medium was examined at rest and in the presence of collagen. Aβ released into the medium was measured using a commercially available human Aβ ELISA kit. **b** Platelets were pre-incubated with β-sitosterol (15 μM) at 37 °C for 2 h followed by simultaneous challenge with cholesterol (2 mM) and β-sitosterol (15 μM) at 37 °C for another 24 h. As a control, platelets were also pre-incubated with vehicle at 37 °C for 2 h followed by challenge with cholesterol (2 mM) and vehicle at 37 °C for another 24 h. Subsequently, platelets were collected and re-suspended in modified Tyrode buffer. Platelet Aβ release in the medium was examined at rest and in the presence of collagen. Aβ released into the medium was measured using the same ELISA kit. All the values are presented as mean±SD ($n=12$). * $P<0.01$ versus corresponding control group (vehicle-only); # $P<0.01$ versus 2 mM cholesterol-treated group

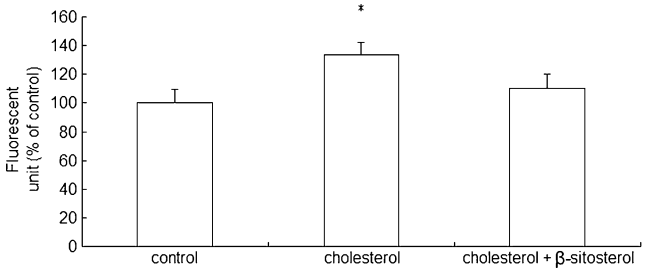

Fig. 2 β-sitosterol inhibits high cholesterol-induced increased β-secretase activity. Platelets were pre-incubated with β-sitosterol (15 μM) at 37 °C for 2 h followed by simultaneous challenge with cholesterol (2 mM) and β-sitosterol (15 μM) at 37 °C for another 24 h. After incubation, platelets were collected by centrifugation and β-secretase activity in platelet pellets was tested by fluorescent spectroscopy using a β-secretase activity detection kit. Values are presented as mean±SD (n=12). * P<0.01 versus control group (vehicle-only)

effectively suppressed high cholesterol-induced increase of β- and γ-secretase activities (P<0.01, Figs. 2 and 3).

β-sitosterol inhibits high cholesterol-induced increase of lipid raft cholesterol

Since membrane cholesterol is closely associated with APP processing (Bouillot et al. 1996; Riddell et al. 2001; Runz et al. 2002; Beel et al. 2010), in this study, we also tested the effects of cholesterol and/or β-sitosterol on levels of lipid raft cholesterol in platelet membrane. It was found that high cholesterol increased levels of lipid raft cholesterol in platelets (P<0.01, Fig. 4). To determine whether β-sitosterol influenced high cholesterol-induced increase of lipid raft cholesterol, we incubated platelets with cholesterol (2 mM) and β-sitosterol (15 μM). We found that β-sitosterol at 15 μM attenuated the high cholesterol-induced increase of lipid raft cholesterol (P<0.01, Fig. 4).

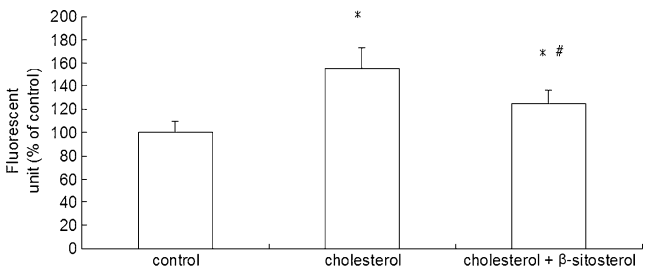

Fig. 3 β-sitosterol inhibits high cholesterol-induced increased γ-secretase activity. Platelets were pre-incubated with β-sitosterol (15 μM) at 37 °C for 2 h followed by simultaneous challenge with cholesterol (2 mM) and β-sitosterol (15 μM) at 37 °C for another 24 h. After incubation, platelets were collected by centrifugation and γ-secretase activity in platelet pellets was tested by fluorescent spectroscopy using a γ-secretase activity kit. Values are presented as mean±SD (n=12). * P<0.01 versus control group (vehicle-only); # P< 0.01 versus cholesterol-treated group

Discussion

Increasing evidence has linked high cholesterol to AD pathogenesis (Wolozin 2001; Miller and Chacko 2004; Puglielli et al. 2005; Xiong et al. 2008). The cholesterol biosynthesis inhibitor statins have been shown to decrease the risk of AD in the elderly (Dufouil et al. 2005; Tang et al. 2006; Cramer et al. 2008; Haag et al. 2009; Li 2010; Haan 2010). In this study, high cholesterol enhanced Aβ efflux from resting platelets and collagen-induced platelets. In addition, two APP processing enzymes, β- and γ-secretase could cleave more substrates in the presence of a high level of exogenous cholesterol, suggesting that cholesterol modulates APP metabolism through increasing the activities of the two enzymes. Since both β- and γ-secretases are located in cholesterol-rich lipid rafts of cell membrane, their activities are vulnerable to cholesterol changes (Xiong et al. 2008). It has been proposed that cholesterol at a certain range of concentrations promotes the formation of lipid rafts for optimal secretase activities (Xiong et al. 2008). This mechanism may be involved in the effect of cholesterol on platelet APP processing. In this study, high cholesterol up-regulated lipid raft cholesterol in platelet membrane. The up-regulation of raft cholesterol suggests increased formation of lipid rafts in the membrane.

Given the association between cholesterol and APP processing, cholesterol may be considered a target for developing new compounds to prevent or treat AD. Plant sterols have the structure very similar to that of cholesterol, and have been widely used to reduce blood cholesterol (Pollack and Kritchevsky 1981; Clifton 2009). Recently, dietary plant sterols have been suggested to be able to pass the BBB and to accumulate in the brain (Field et al. 2004; Jansen et al. 2006; Haag et al. 2009). Therefore, plant sterols can be expected to interfere with cholesterol-depending functions in the central nervous system, including the role of cholesterol in APP metabolism. In this study,

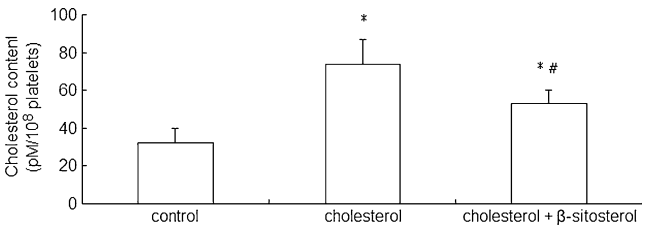

Fig. 4 β-sitosterol inhibits high cholesterol-induced increase in lipid raft cholesterol. Platelets were pre-incubated with β-sitosterol (15 μM) at 37 °C for 2 h followed by simultaneous challenge with cholesterol (2 mM) and β-sitosterol (15 μM) at 37 °C for another 24 h. After incubation, platelets were collected by centrifugation and levels of lipid raft cholesterol were measured by fluorescent spectroscopy using an Amplex red cholesterol assay kit. Values are presented as mean±SD (n=12). * P<0.01 versus control group (vehicle-only); # P<0.01 versus cholesterol-treated group

we showed that β-sitosterol, one of the most common forms of plant sterols, effectively inhibited high cholesterol-induced platelet Aβ efflux and increase of β- and γ-secretase activities. In addition, β-sitosterol treatment inhibited high cholesterol-induced increase in lipid raft cholesterol, suggesting that β-sitosterol inhibited high cholesterol-induced Aβ release possibly through modulation of membrane cholesterol composition.

The mechanisms underlying the effect of β-sitosterol on membrane cholesterol composition are unclear. It has been demonstrated that sitosterol can incorporate into the plasma membrane without affecting membrane fluidity (Mora et al. 1999). Due to the structural similarity between β-sitosterol and cholesterol, β-sitosterol might compete with cholesterol to incorporate into platelets. A previous study showed that phytosterol inhibited the incorporation of exogenous cholesterol into cells at a molar ratio of 1:4 (cholesterol/phytosterol) (Rozner et al. 2008). However, the physiologically achievable plasma concentrations of β-sitosterol (in the micromolar range) are much lower than those of cholesterol (in the millimolar range). In this study, β-sitosterol at micromolar concentrations inhibited millimolar concentrations of exogenous cholesterol-induced increase of lipid raft cholesterol. It sees unlikely that β-sitosterol at this low concentration can competitively inhibit the incorporation of millimolar concentrations of cholesterol into the cells. β-sitosterol may promote cholesterol efflux as a signaling molecule. Recent evidences suggested that β-sitosterol modulated membrane cholesterol composition via acting upon the sterol-responsive nuclear liver X receptor α (LXRα) (Kim et al. 2008), which is known to promote cholesterol efflux via up-regulation of a downstream cholesterol efflux regulatory protein, the ATP-binding cassette transporter ABCA1 (Panzenboeck et al. 2002). However, platelets have no nuclei, which may restrict the effect of β-sitosterol on platelet cholesterol. This might partly explain why β-sitosterol alone did not affect platelet Aβ release. β-sitosterol might modulate intracellular cholesterol transport as a signaling molecule. Normally, blood cholesterol is transported to cells within LDL. Within cells, the cholesterol can be released from LDL in lysosomes and used for membrane biosynthesis. Therefore, modulation of cholesterol circulation between lysosome and plasma membrane may lead to modification of membrane cholesterol composition (Lange et al. 1998). It has been demonstrated that certain amphiphiles (e.g., U18666A and progesterone) can cause the accumulation of lysosomal cholesterol and promote the formation of lysosomes modified for lipid storage, i.e. lamellar bodies (Lange et al. 1998). Since β-sitosterol is an amphiphilic molecule, it is possible that β-sitosterol inhibits exogenous cholesterol-induced increase of lipid raft cholesterol through modulation of cholesterol circulation between lysosome and plasma membrane. However this possibility should be further investigated.

In summary, this study demonstrated that β-sitosterol effectively inhibited high cholesterol-induced platelet Aβ release and increase of β- and γ-secretase activities. In addition, high cholesterol up-regulated lipid raft cholesterol. This effect of cholesterol could also be suppressed by β-sitosterol. These results suggest that β-sitosterol is able to inhibit high cholesterol-driven platelet Aβ release probably through maintenance of membrane cholesterol homeostasis. As there are evidences suggesting that dietary plant sterols have the ability to pass the BBB, plant sterols such as β-sitosterol may be useful in AD prevention.

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