Dietary intake of plant sterols stably increases plant sterol levels in the murine brain

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Abstract

Plant sterols such as sitosterol and campesterol are frequently administered as cholesterol lowering supplements in food. Recently, it has been shown in mice that, in contrast to the structurally related cholesterol, circulating plant sterols can enter the brain. We questioned whether the accumulation of plant sterols in murine brain is reversible.

After being fed a plant sterol ester enriched diet for a period of six weeks, C57BL/6NCrl mice displayed significantly increased concentrations of plant sterols in serum, liver and brain 2- to 3-fold. Blocking intestinal sterol uptake for the next six months feeding the mice with a plant stanol ester enriched diet resulted in strongly decreased plant sterol levels in serum and liver, without affecting brain plant sterol levels. Relative to plasma concentrations, brain levels of campesterol were higher than sitosterol, suggesting that campesterol traverses the blood-brain barrier more efficiently. In vitro experiments with brain endothelial cell cultures showed that campesterol crossed the blood-brain barrier more efficiently than sitosterol. We conclude that, over a 6-month period, plant sterol accumulation in murine brain is virtually irreversible.
Introduction

Plant sterols differ structurally from cholesterol by an additional methyl or ethyl group at C24 and/or a double bond at C22 ($\Delta 22$). The most prevalent plant sterols are campesterol (methyl group at C24), sitosterol (ethyl group at C24), brassicasterol (methyl group at C24, $\Delta 22$), and stigmasterol (ethyl group at C24, $\Delta 22$) (1, 2). In contrast to cholesterol, these sterols are exclusively derived from the diet and cannot be synthesized endogenously in mammals. A high plant sterol intake (2-2.5g/day) leads to reduced total and low density lipoprotein-cholesterol (LDL-cholesterol) (~12%) in the circulation (3, 4). Therefore, plant sterols are frequently applied as functional, non-prescription, food additives to prevent atherosclerosis and cardiovascular diseases (CVD). However, administration of high-dose plant sterols results in increased serum plant sterol concentrations (6). Hard end-point studies, showing an effect on the number of cardiovascular events or on mortality after longterm intake of high-dose plant sterols, are lacking (7) as are insights in the underlying molecular mechanisms (8). Because adverse events upon plant sterol administration in animal studies are increasingly being reported to cause adverse events (9) it is a current topic of debate (10).

In contrast to peripheral tissues, all cholesterol within the central nervous system (CNS) is synthesized in situ since circulating cholesterol is not able to cross the blood-brain barrier (BBB) (11, 12). Recently, we reported that circulating plant sterols, in contrast to cholesterol, can enter the brains of ATP binding cassette g5 (Abcg5) deficient mice, a model for phytosterolemia (13). ABCG5 and ABCG8 act as functional heterodimer transporters at the apical membranes of enterocytes and hepatocytes, where they excrete plant sterols into the intestinal lumen and bile, respectively (14). However, some plant sterols still end up in the serum and small amounts reach the brain.

Accumulating evidence suggests a key role for disturbed brain cholesterol homeostasis in neurodegenerative diseases such as Alzheimer's disease (15-17). A reduction in total membrane cholesterol concentrations, particularly in lipid rafts, has been shown to reduce the production of
amyloid-β (Aβ) by disrupting cleavage of its larger amyloid precursor protein (APP) by the γ-secretase complex (18, 19). Consequently, mild cholesterol depletion impairs APP trafficking to the membrane, making it less prone to cleavage within the lipid rafts or detergent-resistant membranes (DRMs) (18). Moreover, processing of APP into amyloid β (Aβ) requires cholesterol rich DRM microdomains (20). Plant sterols are known to be organized in lipid rafts within plant cell membranes, but effects on the processing of APP remain to be established (21-23). In the present study, we first assessed if plant sterols that had accumulated in the brain are secreted into the circulation by depleting the blood of plant sterols by feeding mice a plant stanol enriched, plant sterol depleting diet. Subsequently, we investigated the redistribution of plant sterols across membranes of different brain cell types.
Materials and Methods

Animals

All animal experiments were approved by the local ethical committee for animal experiments of the Universitätsklinikum des Saarlandes according to institutional and governmental guidelines under project ID: K 110/180-07 19/05. C57BL/6NCrl male mice were purchased from Charles River Laboratories GmbH (Sulzfeld, Germany). At 12 weeks (time point 1), mice (n=29) were randomly divided into two groups (Fig. 1). One group was fed a 2% (w/w) plant sterol ester (PSE) enriched diet (60% sitosterol, 30% campesterol, and 10% stigmasterol; n=14), whereas the other group was fed a control diet (containing 0.015% (w/w) plant sterol esters: 65% sitosterol, 30% campesterol, and 5% stigmasterol). Both diets contained 0.0015% (w/w) plant stanol esters (55% sitostanol and 45% campestanol; n=15). After six weeks of diet, six animals per group were sacrificed (time point 2). Next, the remaining animals in the group previously fed a plant sterol ester diet (n=8) were now fed a 1.5% (w/w) plant stanol ester enriched diet (85% sitostanol, 15% campestanol). The plant stanol ester enriched diet was administered in order to inhibit intestinal sterol uptake and consequently to almost completely deplete the serum of plant sterols. The diet of the remaining animals in the control group (n=9) remained unchanged for a further six months (time point 3; T3). All animals were sacrificed at the end of the experiment with an overdose of ketamine (1g/kg/body weight) and xylazine (100 mg/kg/body weight). Liver and brain hemispheres were snap frozen in liquid nitrogen and stored at -80°C before further analysis. Blood was allowed to coagulate at room temperature for 1h. Serum was subsequently obtained by centrifugation at 4°C for 10 min at 200g. All dietary supplemented sterols and stanols (Raisio Research laboratories, Finland) were mixed in standard rodent chow (Ssniff Spezialdiäten GmbH, Soest, Germany). Dietary sterol concentrations were verified by gas chromatography – mass spectrometry (GC-MS).
Chemical reagents
d6-Sitosterol/d6-campesterol (55%-45%) (Sugaris GmbH, Münster, Germany), d6-cholesterol (99.0%) (CDN isotopes, Pointe-Claire, Quebec, Canada), and epi-coprostanol (99.9%) (Sigma-Aldrich Chemie, Steinheim, Germany) were quantified and tested for purity against 5α-cholestane (1µg/µl) (Serva Electrophoresis GmbH, Heidelberg, Germany) using gas chromatography-flame ionization detection (GC-FID) and GC-MS scan methods. Sterols were dissolved in 100% ethanol to obtain 10mM stock solutions.

Cell culture experiments
Human astrocytoma CCF-STTG1 and human neuroblastoma SHSY5Y cell lines, purchased from the European Collection of Cell Cultures (CACC, Salisbury, UK) were cultured in 10% FCS (EU approved origin, Gibco, Invitrogen GmbH, Karlsruhe, Germany) containing 1:1 Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 Nutrient mixture (DMEM/F-12) (Gibco, Invitrogen GmbH, Karlsruhe, Germany) including 100 units penicillin and 100µg streptomycin per ml (pen/strep from Gibco, Invitrogen GmbH, Karlsruhe, Germany). Immortalized human adult brain endothelial cells hCMEC/D3 cells were obtained from Weksler et al. (24). OLN-93 cells (oligodendroglioma cells derived from primary rat brain glial cultures) were kindly provided by Dr. Richter-Landsberg (25). Primary astrocytes were isolated from human brain as described (26) and applied in the experiments between passage 25 and 30.

In vitro BBB-model
The human brain endothelial cell line (hCMEC/D3) was used as an in vitro BBB-model (24). hCMEC/D3 cells display predominant features of BBB endothelial cells including reduced paracellular permeability. hCMEC/D3 were seeded on collagen-coated Costar Transwell filters
(pore-size 0.4 µm; Corning Incorporated) in growth medium containing 2.5% FCS and were cultured for four days (200µl medium in the apical compartment, 800µl in the basolateral compartment) (24). Subsequently, 15 µM d6-sitosterol/d6-campesterol or d6-cholesterol was added to the apical compartment (blood side) of the Transwell filter setup and incubation was continued for 24h. Cellular sterol uptake (%) was calculated as 
\[
\frac{([d6-sterols \text{ in endothelial cells}] + [d6-sterols \text{ at brain side}])}{([d6-sterols \text{ at blood side}] + [d6-sterols \text{ at brain side}] + [d6-sterols \text{ in endothelial cells}] )} \times 100\%.
\]
Basolateral flux (%) was calculated as 
\[
\frac{[d6-sterols \text{ at brain side}]}{([d6-sterols \text{ at brain side}] + [d6-sterols \text{ in endothelial cells}] )} \times 100\%.
\]
D6-sterols present in astrocytes were included in the “brain side” for calculations.

Astrocyte conditioned medium has been shown to enhance maturation of endothelial cells, further strengthening endothelial tight junctions and restricting the permeability of the endothelial monolayer, thus improving the BBB model (27). Therefore, the trans-endothelial flow experiment was repeated with primary astrocytes cultured to confluency at the bottom of the basolateral compartment in the endothelial-Transwell setup. D6-sterols were measured after sterol extraction by applying GC-MS analysis.

To show that addition of sterols did not influence cellular permeability, permeability was measured as described previously (27). In short, after 23 h of culturing and one hour before harvesting of the cells, fluorescein isothiocyanate coupled dextran (FITC-dextran: 150 kDa, 1µg/µl in culture medium; Sigma-Aldrich) was added to the apical compartment as described previously (28). One hour later, 10µl sample was collected from the apical and basolateral compartment for measurement of fluorescence intensity using a FLUOstar Galaxy microplate reader (BMG Labtechnologies; excitation 485nm and emission 520nm, at gain 64). The average 1h flux of FITC-dextran through the endothelial monolayer was calculated and extremes were excluded from further analyses based on Dixon’s principles of exclusion for extreme values (29, 30).
Viability of the hCMEC/D3 cells, determined by Water-Soluble Tetrazolium (WST) assay, was found to be unaltered after incubation with different concentrations of d6-sitosterol/d6-campesterol or d6-cholesterol (0, 5, 10, 15, 20, 25 and 30µM) after different incubation times (6h, 24h and 48h) (data not shown).

Efflux studies

CCF-STTG1 (astrocytes), SHSY5Y (neurons) and OLN-93 (oligodendrocytes) cells were cultured in DMEM/F12 medium (Gibco, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% FCS until 90% confluency. After washing cells with PBS, cells were incubated with medium containing 0.5% FCS and 15µM d6-cholesterol or d6-sitosterol/d6-campesterol in the presence of 1µM T0901317, a synthetic Liver X Receptor-agonist that is known to enhance cholesterol efflux from cells (31). After 24h, the medium was discarded. Cells were washed thrice with PBS. Next, serum free medium containing either 25µg/ml HDL (isolated as described previously (32) from pooled serum of four healthy individuals) as a sterol-acceptor or vehicle control (0.2% BSA in PBS) was added and cells were incubated for a period of 6h in the presence of 1µM T0901317. Medium and cells were collected separately and analyzed by GC-MS for d6-cholesterol and d6-sitosterol/d6-campesterol content, respectively.

Detergent Resistant Membrane isolation from confluent cell lines

SHSY5Y cells were cultured in T-75 flasks until confluency in medium containing 10% FCS. Cells were then incubated in medium containing 0.5% FCS and 15µM of d6-sitosterol/d6-campesterol, d6-cholesterol (10 mM stock) or vehicle (100% ethanol) for a period of 24h. Lipid rafts or detergent-resistant membranes (DRMs) were isolated as described previously (33). In brief, cells were washed twice with ice cold PBS. Next, cells were scraped in 1.5ml ice cold TNE buffer (25mM Tris, pH 7.4, 150mM NaCl, and 2mM EDTA) containing Complete protease inhibitor mix (Roche Diagnostics
GmbH, Mannheim, Germany), and disrupted by 15 strokes through a 25-G needle. Nuclei were precipitated by low speed centrifugation (20min, 1,000g, 4°C) and discarded. Membranes were precipitated from the supernatant by high speed centrifugation (90min, 17,000g, 4°C). The membrane pellet was then dissolved in 1.5ml 1% CHAPSO (Roche Diagnostics GmbH, Mannheim, Germany) containing Complete protease inhibitor mix and incubated on ice for 30 min. The CHAPSO cell extracts were mixed with 2.5ml TNE buffer containing 72% sucrose to yield a final concentration of 45% (w/v) sucrose and placed at the bottom of 12ml Beckman ultracentrifuge tubes. Four ml of TNE buffer containing 35% and 5% sucrose were successively and carefully layered on top of the CHAPSO cell extracts. The samples were spun at 4°C for 19h at 40,000 rpm in a SW41 TI rotor (Beckman Coulter GmbH, Krefeld, Germany). One ml fractions were carefully collected from top to bottom to yield a total of 12 fractions. The low buoyant DRM fraction was positioned at the interface between the 35% and 5% sucrose as amorphous white material visible to the naked eye. It was collected in fractions 4 and 5. DRMs (density: 1.09-1.13g/cm³ (34)) were confirmed by densitometry using a DMA48 density meter (Anton Paar GmbH, Graz, Austria), by flotillin-1 expression on western blotting, and cholesterol content by GC/MS (Fig 4 E-F). The non-DRM fractions were confirmed to be in fractions 8-10. All separate fractions were subjected to sterol extraction preceding GC-MS analysis.

Preparation of total membrane fraction of mouse brain hemispheres

Snap frozen wet mouse brain hemispheres were weighted (mg) prior to homogenization as described previously (35). 1.5ml homogenization buffer (150mM NaCl, 20mM Na₂HPO₄, 2mM NaH₂PO₄, 1mM EDTA, and 20% (vol/vol) glycerol, pH 7.4) was added to each hemisphere (36). Two cycles of 30s at 6,500rpm in the Precellys24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) were run, separated by 5min of cooling on ice. Total homogenate was used to measure protein concentrations (Biorad DC protein assay kit). After homogenization, the samples were
diluted 1:4 in homogenization buffer and centrifuged at 5,000g for 20min. The pellet was discarded and the supernatant was centrifuged 90min at 17,000g. The resultant membrane pellet was resuspended in 1.5ml TNE buffer containing 1% CHAPSO (Roche Diagnostics GmbH, Mannheim, Germany) and incubated on ice for 30min. DRMs were isolated by density gradient centrifugation, and characterized as described above for the confluent cell lines.

**Sterol profile determination**

Wet weights of hemispheres and protein concentrations of brain homogenates were determined in order to relate sterol content to wet weight or protein concentration. Sterol contents of fractions generated from brain homogenates, total brain homogenates, cell lysates, fractions generated by sucrose gradient ultracentrifugation, total medium and cell lysates from the efflux experiment were determined by GC-MS as previously described (37, 38).

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 4™. The sterol data in serum, liver and brain were analyzed using the 1-way ANOVA with a post hoc Bonferroni’s Multiple Comparison Test. Extreme values were excluded by means of Dixon’s principles of exclusion of extreme values (29, 30). Membrane fraction sterol concentrations were compared by 2-way ANOVA with fraction and treatment as variable factors (post hoc Bonferroni’s Multiple Comparison Test). Significance levels were determined on #, §, *: P<0.05, ##, §§, **: P<0.01 or ###, §§§, ***: P<0.001.
Results

Dietary administration of plant sterols stably increased brain plant sterol levels

C57BL/6NCrl mice were either fed a normal chow diet (NC) (n=15) or were given a 2% plant sterol ester enriched diet (PSE) (n=14) for a period of six weeks (Fig. 1). In line with several previous reports (13, 39), the PSE fed group displayed significantly increased sitosterol and campesterol concentrations in serum, liver and brain in comparison to the NC group (Fig 2A-C). When related to their serum concentrations, the enrichment of campesterol in the brain was 1.2-fold higher than sitosterol.

In the PSE-fed group, feeding the mice a 1.5% plant stanol ester enriched diet (PSA) for a period of six months significantly reduced sitosterol and campesterol concentrations in serum (sitosterol: P<0.001 and campesterol: P<0.001; Fig. 2A) and liver (sitosterol: P<0.001 and campesterol: P<0.001; Fig. 2B). However, brain plant sterol concentrations remained unchanged despite almost complete depletion of circulating plant sterols (Fig. 2C). Brain cholesterol and sitostanol were not affected by the different diets, while brain campestanol increased twofold after administration of the PSA diet, albeit remaining 300-fold lower in concentration compared to campesterol (data not shown). Also relative to the cerebral cholesterol content, the amount of cerebral plant sterols did not decrease in the PSE fed group following administration of the PSA diet for a period of six months ([campesterol(ng)/cholesterol(µg)]: 2.36±0.18 (PSE) vs 2.65±0.31 (PSA), n.s. and [sitosterol(ng)/cholesterol(µg)]: 0.58±0.03 (PSE) vs 0.53±0.11 (PSA), n.s.). Animals fed a NC diet displayed a modest increase in the campesterol to cholesterol ratio and a comparable sitosterol to cholesterol ratio over a period of 6 months ([campesterol(ng)/cholesterol(µg)]: 1.10±0.05 (NC 6 weeks) vs 1.30±0.05 (NC 6 months), P<0.05 and [sitosterol(ng)/cholesterol(µg)]: 0.34±0.01 (NC 6 weeks) vs 0.31±0.05 (NC 6 months), n.s.).
Together, these results strongly suggest that plant sterols enter the brain depending on the concentration in the serum and accumulate stably in the brain, despite the fact that plant sterol concentrations in serum were strongly reduced during the six months PSA diet.

**Campesterol is more efficient than sitosterol in traversing a brain endothelial monolayer**

In order to examine whether sitosterol, campesterol, and/or cholesterol traverse the endothelial cell layer of the BBB or accumulate within these cells, d6-sitosterol/d6-campesterol or d6-cholesterol were added to the apical side of a polarized confluent human brain endothelial hCMEC/D3 monolayer in an in vitro Transwell® setup (Fig. 3A). After 24h, the internalization of all sterols was comparable (Fig. 3B). However, the amount of d6-cholesterol in the basolateral compartment (0.97%±0.10 of uptake) was approximately five times higher than d6-sitosterol and d6-campesterol (Fig. 3C). Despite a comparable apical uptake of d6-sitosterol and d6-campesterol, d6-campesterol was 1.4-fold higher in the basolateral compartment compared to d6-sitosterol (0.22%±0.04 vs 0.15%±0.03; P=0.016; Fig. 3B). Using endothelial monolayers cultured in the presence of astrocytes we found that the absolute amount of d6-sterols was increased about 2-fold in the basolateral compartment (Fig. 3D). One third of the d6-sterols in the lower compartment was incorporated by the astrocytes (d6-cholesterol: 40±7%, d6-campesterol: 34±6% and d6-sitosterol: 29±6%). These data suggest that passage of the sterols across the endothelial monolayer depends on the molecular complexity of their side chain.

**Dietary administration of plant sterols increased brain plant sterol concentrations predominantly in detergent resistant membranes (DRM).**

Considering the functional importance of cholesterol in neuronal membranes, the distribution of plant sterols within the membranes was determined. Membranes were isolated from brain homogenates of mice fed for six weeks with a PSE enriched or a NC diet. Higher levels of plant
sterols in DRM than in the non-DRM fractions (sitosterol NC: DRM vs non-DRM: P<0.001, sitosterol PSE: DRM vs non-DRM P<0.001, campesterol NC: DRM vs non-DRM: P<0.001 and campesterol PSE: DRM vs non-DRM P<0.001; Fig. 4A-D) were observed irrespective of the diet (NC or PSE). Moreover, the PSE diet induced a significantly higher accumulation in the DRM fractions, but not in the non-DRM fractions, in comparison with the NC diet (sitosterol DRM: NC vs PSE: P<0.05 and campesterol DRM: NC vs PSE: P<0.001; Fig. 4A-D). In contrast, the amount of cholesterol, and the distribution over DRM and non-DRM fractions was not affected by the diet (Fig. 4E-F). Therefore, these data show that plant sterols accumulate within the DRM fractions of the brain cell membranes dependent on the plant sterol concentrations in the brain.

Plant sterols accumulate in detergent-resistant membranes in cultured brain cells

Plant sterol and cholesterol uptake and efflux to HDL were determined using different human brain derived cell lines. Neuronal cells incorporated significantly less plant sterols and cholesterol than glial cells (oligodendrocytes and astrocytes) both after 6h and 24h of incubation (Table I). Slightly, but not significantly, more cholesterol than plant sterols was taken up by all cell lines. In contrast to glial cells, sterol efflux in the neuronal cell line could not be enhanced by adding external HDL as acceptor. The efflux of plant sterols was slightly higher than of cholesterol. However, when the percentages were corrected for absolute uptake values, absolute amount of cholesterol and plant sterols was comparable.

After administration of 10µM d6-sitosterol/d6-campesterol (55%-45%) mixture to SHSY5Y cells, both d6-sitosterol (P<0.001) and d6-campesterol (P<0.001) were found to accumulate preferentially in the cholesterol rich DRMs rather than in the phospholipid enriched non-DRM fractions (Fig. 4G). The ratio of both plant sterols of the administered mixture was maintained within each fraction measured and the accumulation paralleled the accumulation of administered d6-cholesterol (data not shown).
In conclusion, these data show that two different plant sterols and cholesterol are handled similarly by the individual cerebral cell lines and subsequently accumulate within the DRMs.
Discussion

We demonstrate for the first time that increased dietary intake of plant sterols results in a concentration-dependent, almost irreversible accumulation of plant sterols within the murine brain. In addition, we showed, both in vitro and ex vivo, that plant sterols preferentially accumulate within DRMs of brain cells.

Previously, we reported that dietary derived plant sterols can enter the brain (13, 39). Our present data confirm that cerebral plant sterol concentrations significantly increase after six weeks of dietary plant sterol supplementation, with a selective advantage for campesterol. More importantly, six months following plant stanol ester supplementation plasma and liver were almost depleted of plant sterols, whereas plant sterol concentrations in the brain remained unaffected, indicating an almost irreversible accumulation of the plant sterols. Plant stanol treatment was used because of its very low intestinal absorbability compared with the pharmaceutical sterol uptake blocker ezetimibe (40). Direct influences of ezetimibe on sterol transport at the blood-brain barrier cannot be excluded. Although we did not run a time-course experiment, it is possible to estimate the rate of sterol accumulation in the brain from our previous studies. We showed that 15 week old Abcg5−/− mice have plasma campesterol concentrations of 10 mg/dl, resulting in 6.43 mmol campesterol/mol cholesterol in the brain (13). In the current experiment, C57BL/6Ncrl mice were fed a 2% enriched plant sterol diet for six weeks similarly resulting in comparable plasma campesterol concentrations of 10mg/dl, and brain campesterol concentrations of 2.28 mmol campesterol/mol cholesterol. Three month old Abcg5−/− mice were exposed 2.5 times longer to the same 10mg/dl plasma campesterol concentrations, corresponding to a parallel 2.8 fold higher cerebral campesterol concentrations. Although this is only a theoretical calculation, it supports a linear time-dependent uptake of plant sterols in the brain. Importantly, the Abcg5 transporter is not detectable within the brain and therefore is unlikely to influence plant sterol transport across the BBB (13). Given the assumption
that residual plasma volume is 10 μl per gram of brain tissue, and that human brain contains about 100 mg protein per gram wet weight, together with the data shown in Fig. 2C, it would seem that less than 5% of sitosterol and campesterol in the brain is contained within residual serum of mice on PSE fed.

In vivo, based on plasma concentrations, campesterol accumulated to a relatively higher amount (1.2 fold) in the brain compared to sitosterol, and in vitro campesterol transfer across a hCMEC/d3 monolayer was 1.4-times more efficient than for sitosterol. The two-fold increase in flow of sterols across the endothelial cell monolayer in the presence of primary astrocytes at the bottom of the lower chamber might involve the secretion of sterol accepting lipoprotein like particles by the astrocytes. Therefore, both endothelial cells and astrocytes may be involved in plant sterol transport across the BBB. Compared with plant sterols, we found a five-fold higher secretion of the apically internalized cholesterol at the basolateral side after 24h incubation. Considering the large pool of endogenous cholesterol within the cell, the total amount of cholesterol released at the basolateral side is probably even higher. Thus, trans-endothelial flux efficiency is determined by the molecular complexity of the sterol side-chain. A possible explanation for this may be a difference in the esterification rate within the endothelial cells. Like acyl-CoA:cholesterol O-acyltransferase (ACAT)2, which is mainly expressed in enterocytes, it has been shown that ACAT1, which is ubiquitously expressed, esterifies cholesterol more efficiently than sitosterol, independent of ACAT1 protein expression (2, 41, 42). However, the ratio of free/total cholesterol in hCMEC/D3 cells was >99%, making it difficult to reliably detect differences in esterification between cholesterol and plant sterols. It is generally assumed that the brain synthesizes all of the required cholesterol in situ and is therefore independent of peripheral cholesterol supply (11, 43). However, several in vitro and in vivo studies demonstrated a small, but significant transport of cholesterol across the BBB. Administration of 4-C\(^{14}\)-cholesterol to terminally ill patients revealed an average cerebral cholesterol accumulation of 3.2% of the serum specific activity levels 1 to 244 day after injection (44). However, a defective BBB could not be
excluded in these terminally ill patients. Administration of d6-cholesterol in an adult guinea pig resulted in a 1.23% and 0.93% accumulation in cerebrum and cerebellum, respectively (45). In addition, feeding of 0.5% d6-cholesterol to mice and rats for a period of ten days resulted in cerebral accumulation of less than 1% in either species (46). An in vitro porcine brain endothelial cell monolayer Transwell setup revealed an approx. 2% apical flux of cholesterol after basolateral administration (47). In our experiments, the basolateral uptake of plant sterols and cholesterol over a period of 24h was very limited (campesterol: 0.26%, sitosterol: 0.21% and cholesterol 3.68% compared to the common 52% uptake at the apical side; data not shown). The subsequent apical amount was only a very limited percentage of the absorbed sterols (campesterol: 4.74% sitosterol: 1.83% and cholesterol: 2.32%; data not shown), making the absolute flux more efficient from apical to basolateral side than the reverse transport. Together, these data all support a small but significant flux of cholesterol across the BBB. Due to the high cerebral cholesterol content and tightly regulated cholesterol turnover, the amount of cholesterol crossing the BBB is expected to be negligible and to be balanced by cerebral cholesterol homeostatic mechanisms. Under non-diseased conditions, little cholesterol from the circulation enters the brain. However, this may be different under diseased conditions with impaired BBB. In the human brain, cholesterol is metabolized into brain-specific 24(S)-hydroxycholesterol in order facilitate cerebral cholesterol efflux, accounting for 60% of cholesterol release (48-52). Although plant sterols cross the endothelial monolayer less efficiently than cholesterol, they cannot be metabolized into 24(S)-hydroxysterols. 24-hydroxylation of plant sterols by the highly specific cholesterol-24-hydroxylase, encoded by CYP46A1, is prevented because of steric hindrance by the alkyl group at C24 (53). Furthermore, hydroxylation of plant sterols at position 7α in order to metabolize 24-alkyl plant sterols is very unlikely since 24(S)-hydroxycholesterol, in contrast to 27-hydroxycholesterol and 25-hydroxycholesterol, is not 7α hydroxylated by cultured astrocytes, Schwann cells, or neurons (51, 54). The inability to convert plant sterols into more polar hydroxysterols explains why plant sterols, once having crossed the
BBB, cannot easily leave the brain, leading to their persistent accumulation as described in this study. However, a very small, but non-significant reduction of cerebral sitosterol (20%; P=0.15) and campesterol (3%; P=0.81) was observed after a six month PSA enriched diet. Since the brain sterol turnover is known to be extremely slow, a low rate of plant sterol efflux cannot be excluded over longer time intervals.

Our previous studies indicated that plant sterol accumulation in the brain of Abcg5-deficient mice affected cerebral cholesterol homeostasis, since levels of desmosterol and 24(S)-hydroxycholesterol in the hippocampus were decreased and lanosterol levels in the cortex were increased. This was not associated with alterations in learning and memory functions of the Abcg5-deficient mice (55). However, plant sterol accumulation may have consequences for brain function in neuropathological diseases.

Our in vitro endothelial monolayer Transwell system shows that plant sterols are transported rather slowly through endothelial cells of the vessel walls. It should be taken into account that plant sterol accumulation in endothelial cells may impair endothelium-dependent vasorelaxation and increase cerebral lesion size after middle cerebral artery occlusion followed by reperfusion (9, 56). The mechanisms by which plant sterols are taken up by cerebral endothelial cells have not been revealed to date. Lipoprotein receptors like the low density lipoprotein receptor (LDLR) and scavenger receptor BI (SRBI) are expressed by cerebral endothelial cells (57, 58). Comparative studies with LDLR knockout and wildtype mice revealed that LDLR is not involved in cerebral plant sterol uptake (unpublished observations). Plasma plant sterols are mainly transported in HDL particles. Similar to Abcg5 knockout mice, ApoE knockout mice display high plasma plant sterol concentrations. However, in contrast to Abcg5 knockout mice, ApoE knockout mice display low HDL related plant sterol concentrations, resulting in relatively strongly reduced cerebral plant sterol concentrations (13). Therefore, a HDL receptor such as SRBI might be a suitable candidate for plant sterol uptake by endothelial cells in the brain. Our observation that plant sterol levels are increased
to the same extent in Abcg5 knockout mice and in Abcg5 knockout mice that also lack the SRBI, suggests that the SRBI is not involved in the transport of plant sterols across the BBB (unpublished observations).

We demonstrated that plant sterols reside in lipid rafts within the brain cell membranes. Moreover, plant sterols reduce molecular order and fluidity of membranes by interacting less efficiently with saturated phospholipids than with cholesterol as is required in the formation of compact, liquid ordered lipid rafts (59, 60). It was demonstrated that the magnitude of the effect on membrane fluidity depends on the geometry of the sterol molecule that is determined by the structure of its side chain (cholesterol >> campesterol > sitosterol > stigmasterol) (59). Mild reductions in membrane cholesterol impact on the cleavage of APP upstream of γ-secretase and is mediated by changes in APP trafficking and partitioning into lipid rafts (18). Therefore, incorporation of plant sterols in lipid rafts potentially influences γ-secretase mediated cleavage of APP in lipid rafts. The influence of plant sterol accumulation on Alzheimer’s disease pathology remains to be determined.

In conclusion, these data show that long term consumption of large amounts of plant sterols results in virtually irreversible accumulation of plant sterols in murine brains. Together with our in vitro results, these data indicate the need for further studies into the influence of long term plant sterol intake on normal and pathological brain function.

Acknowledgements

We thank Prof. Dr. J. Dietschy, Prof. Dr. I. Björkhem, Dr. P. Delhanty and Dr. A. J. Verhoeven for critical remarks and advice. All experimental procedures with mice were performed according to German governmental guidelines. There are no conflicts of interest. The research leading to these results has received funding from the EU FP7 project LipiDiDiet (FP7/2007-2013; grant agreement n° 211696) and Marie Curie Early Stage Training Fellowships (grant agreement n° MEST-CT-2005-02058).
References


**Legends to figures**

**Figure 1.** Experimental scheme of C57BL/6NCrl feeding experiment

At 12 weeks (T₁), C57BL/6NCrl (n=29) were randomly subdivided into two groups fed either a regular chow diet (n=14) or a plant sterol ester enriched diet (PSE) (n=15). After six weeks (T₂), six animals per group were sacrificed. The remaining animals on the regular chow diet (n=9) continued to be fed the normal chow diet. The remaining mice from the PSE group (n=8) were then fed a plant stanol ester (PSA) enriched diet. After six months of the second feeding term (T₃), all animals were sacrificed.

**Figure 2.** Plant sterol concentrations in serum, liver and brain with or without plant sterol or plant stanol administration.

Twelve week old C57BL/6NCrl mice were fed either a normal chow (NC) (n=15) or a plant sterol enriched chow (PSE) (n=14). After six weeks, six animals per group were sacrificed. The remaining animals on NC (n=9) continued to be fed NC and the animals previously fed PSE (n=8) were given a plant stanol enriched chow (PSA) for six more months. Concentrations of sitosterol and campesterol in serum (mg/dl) (A), liver (ng/mg dry weight) (B), and brain (ng/mg protein) (C) are shown as mean ± SEM. PSE leads to elevated plant sterols in the serum (A), liver (B) and brain (C) after 6 weeks. PSA reduces serum and liver plant sterol, but not brain plant sterol concentrations after 6 months. ANOVA, Bonferroni Post Hoc: “§”: compared to six weeks NC fed C57BL/6NCrl mice and Student t-tests: “#”: PSE versus PSA, “*”: six months PSA versus six months NC. §, *: P<0.05, §§: P<0.01, §§§, ###, ***: P<0.001

**Figure 3.** Apical uptake and subsequent basolateral efflux of plant sterols and cholesterol across a human endothelial cell monolayer.
hCMEC/D3 endothelial cells were grown to confluency on permeable Transwell membranes (A) in the absence (C) or presence (D) of a confluent layer of primary astrocytes (AC) at the bottom of the lower chamber. 15μM d6-sitosterol/d6-campesterol and 15μM d6-cholesterol, respectively, were added to the upper compartment. After 24h, the apical uptake rate (B) and consequent basolateral flux (C & D) were quantified. Uptake (%) was calculated as \[\frac{[(\text{d6-sterols in endothelial cells}) + (\text{d6-sterols at brain side})]}{[(\text{d6-sterols at blood side}) + (\text{d6-sterols at brain side}) + (\text{d6-sterols in endothelial cells})]} \times 100\%\] (B). Basolateral flux (%) was calculated as \[\frac{[(\text{d6-sterols at brain side})]}{[(\text{d6-sterols at brain side}) + (\text{d6-sterols in endothelial cells})]} \times 100\%\] (C & D). D6-sterols present in astrocytes were included in the “brain side” for calculations (D). Flux was significantly higher for d6-cholesterol than for the plant sterols. d6-Campesterol flux was significantly higher than d6-sitosterol. Values represent the mean (%) of six independent experiments ± SD. Please note different scales. **: P<0.01 and ***: P<0.001

Figure 4. Plant sterol concentrations in homogenates and DRM fractions of murine brains, with or without plant sterol or plant stanol administration.

Twelve week old C57BL/6NCrl mice were fed either a normal chow (NC) (n=15) or a plant sterol enriched chow (PSE) (n=14). After six weeks, six animals per group were sacrificed and sitosterol (A), campesterol (B) and cholesterol (C) concentrations were determined in 12 x 1ml (top-bottom) density fractions of the membranes (ng/fraction/mg wet weight). As depicted in the materials and methods section, membrane fractions were subdivided in detergent-resistant membrane fractions (DRMs fractions 4 and 5) and non-DRMs (fractions 8, 9 and 10). Sitosterol (B), campesterol (D) and cholesterol (F) DRM and non-DRM fraction masses were added up. PSE administration leads to significantly elevated plant sterol concentrations in DRMs, but not in non-DRM regions of the brain membranes. The increased accumulation of campesterol and sitosterol in DRM compared to non-DRM was subsequently validated by 24h administration (n=5) of a 10μM d6-sitosterol/d6-
campesterol (55%-45%) mixture to the SHSY5Y neuroblastoma cell line (G). *: DRM compared to non-DRM, §: PSE compared to NC fed C57BL/6/6NCrl mice; §: P<0.05, §§§,***: P<0.001; n.s.: not significant.
Tables

Table I: Uptake and efflux of d6-sitosterol, d6-campesterol and d6-cholesterol for a neuroblastoma, astrocytoma and oligodendroglioma cell line. Uptake is shown after 6 and 24h of incubation. Efflux is shown after 24h of incubation and a subsequent efflux period of 6h during which either HDL was added as an acceptor or 0.2% BSA as a vehicle.

<table>
<thead>
<tr>
<th>UPTAKE (%)</th>
<th>d6-sitosterol</th>
<th>d6-campesterol</th>
<th>d6-cholesterol</th>
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<tbody>
<tr>
<td>6h</td>
<td>24h</td>
<td>6h</td>
<td>24h</td>
</tr>
<tr>
<td>neuron.</td>
<td>4.2 (0.6)</td>
<td>15.5 (1.0)</td>
<td>4.4 (0.6)</td>
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<td>11.1 (0.6)</td>
<td>42.1 (5.7)</td>
<td>10.2 (0.6)</td>
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<td>oligod.</td>
<td>9.8 (0.8)</td>
<td>34.4 (3.0)</td>
<td>10.4 (0.7)</td>
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<table>
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<tr>
<th>EFFLUX (%)</th>
<th>d6-sitosterol</th>
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<td>6h</td>
<td>24h</td>
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<tr>
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<td>16.7 (0.4)</td>
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<td>37.3 (2.9)*</td>
<td>12.7 (3.4)</td>
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<td>oligod.</td>
<td>4.2 (0.7)</td>
<td>5.7 (1.4)*</td>
<td>4.1 (0.7)</td>
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Values are displayed in % ± SD of the amount added to the cells for uptake and % ± SD of the amount that was internalized by the cells after 24h incubation for efflux. *= P<0.05; **=P<0.01 Mann-Whitney U test HDL vs BSA. Data represent the mean of 3 independent experiments
Figure 1 (Vanmierlo et al)

- C57BL/6NCrl (n=15)
  - 6 weeks chow diet
- C57BL/6NCrl (n=14)
  - 6 weeks PSE diet
  - 6 months PSA diet
  - † C57BL/6NCrl (n=8) sacrificed
- C57BL/6NCrl (n=9) sacrificed

* C57BL/6NCrl (n=29)
  - † C57BL/6NCrl (n=6) sacrificed

T<sub>0</sub>: 0w  T<sub>1</sub>: 12 w  T<sub>2</sub>: 18 w  T<sub>3</sub>: 44 w

age
Figure 2 (Vannierio et al.)

**SITOSTEROL**

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<tr>
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<td>PSA</td>
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**CAMPESTEROL**

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<tr>
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<tr>
<td>PSA</td>
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</table>

**A**

**SERUM**

- mg/dL
- NC
- PSE
- PSA

**B**

**LIVER**

- ng/mg dry weight
- NC
- PSE
- PSA

**C**

**BRAIN**

- ng/mg prot
- NC
- PSE
- PSA
Figure 3 (Vanmierlo et al.)

**A**  
Diagram showing the setup for Fig. 3C and Fig. 3D.

**B**  
Graph showing the % sterol uptake in hCMEC/D3 (24h) for sitosterol, campesterol, and cholesterol.

**C**  
Bar graph showing the % flux to brain side hCMEC/D3 uptake for sitosterol, campesterol, and cholesterol.

**D**  
Bar graph showing the % flux to brain side hCMEC/D3 uptake for sitosterol, campesterol, and cholesterol.
Figure 4 (Vanmierlo et al.)

A. Campesterol

B. Campesterol levels comparison between DRM and non-DRM conditions.

C. Sitosterol

D. Sitosterol levels comparison between DRM and non-DRM conditions.

E. Cholesterol

F. Cholesterol levels comparison between DRM and non-DRM conditions.

G. 6β-sitosterol and 6β-campesterol levels comparison between DRM and non-DRM conditions.