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Intake of stigmasterol and β -sitosterol alters lipid metabolism and alleviates NAFLD in mice fed a high-fat western-style diet

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Abstract

Objective: To investigate and compare the effects of two common dietary phytosterols, stigmasterol and β -sitosterol, in altering lipid metabolism and attenuating nonalcoholic fatty liver disease (NAFLD).

Methods: Stigmasterol and β -sitosterol were administered to mice at 0.4% in a high-fat western-style diet (HFWD) for 17 weeks.

Results: Stigmasterol and β -sitosterol significantly ameliorated HFWD-induced fatty liver and metabolic abnormalities, including elevated levels of hepatic total lipids, triacylglycerols, cholesterol and liver histopathology. Both phytosterols decreased the levels of intestinal bile acids, accompanied by markedly increased fecal lipid levels. In addition, they altered the expression of

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genes involved in lipid metabolism. β -Sitosterol was less effective in affecting most of these parameters. Lipidomic analysis of liver and serum samples showed that stigmasterol prevented the HFWD-induced elevation of some di- and triacylglycerol species and lowering of some phospholipid species. Stigmasterol also decreased serum levels of ceramides.

Conclusion: Stigmasterol and β -sitosterol, at a dose corresponding to that suggested for humans by the FDA for lowering cholesterol levels, are shown to alleviate HFWD-induced NAFLD. Stigmasterol was more effective than β -sitosterol, possibly because of its suppression of hepatic lipogenic gene expression and modulation of circulating ceramide levels.

Keywords

stigmasterol; β -sitosterol; fatty liver; mice; cholesterol; lipidomics

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) ¹ is a highly prevalent condition characterized by fatty infiltration of liver cells in the absence of excessive alcohol consumption [1]. It includes a wide spectrum of liver damage stages ranging from isolated hepatic steatosis without liver inflammation to non-alcoholic steatohepatitis (NASH), with the latter being characterized as cryptogenic cirrhosis and even hepatocellular carcinoma [2]. The cellular mechanisms contributing to the progression of NAFLD are not fully understood.

Although hepatic fat accumulation is the hallmark of NAFLD, the hepatic fats that accumulated in the development of NAFLD are not well characterized. It was summarized in a review that disturbed hepatic cholesterol homeostasis and hepatic free cholesterol accumulation contribute to the pathogenesis of NAFLD [3]. Cholesterol-lowering agents, such as atorvastatin and ezetimibe, have been reported as therapeutic tools in the treatment of NAFLD [4]. Some other studies have focused on hepatic triacylglycerol (TG) accumulation as a key factor in NAFLD [5]. Free fatty acids (FFA) were also reported to contribute to the development of NAFLD, as they increase intracellular oxidative stress and cause cell injury [6, 7]. However, the roles of the accumulation of other lipid classes, such as diacylglycerols (DG), phosphatidylethanolamines (PE), sphingomyelins (SM), TG, phosphatidylcholines (PC) and ceramides (CM), in the progression of NAFLD are still not known.

Phytosterols, the plant analogues of cholesterol, are known to decrease the circulating cholesterol levels by competing with cholesterol for intestinal absorption or regulating

¹ABCA1, ATP binding cassette transporter A1; ABCG5, ATP binding cassette transporter 5; ALT, alanine aminotransferase; ASBT, ileal apical sodium-dependent bile acid transporter; BA, bile acid(s); BSEP, bile salt export pump; CD36, Cluster of Differentiation 36; CM, ceramide(s); CYP7A1, cholesterol 7 α -hydroxylase gene; CYP8B1, sterol 12 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; DG, diacylglycerol(s); FAS, fatty acid synthase; FFA, free fatty acid(s); FGF15, Fibroblast growth factor 15; FXR, farnesoid x receptor; HDL-C, high-density lipoprotein cholesterol; HFWD, high-fat western-style diet; HMGCoAR, hydroxymethylglutaryl coenzyme A reductase; non-HDL-C, non-high-density lipoprotein cholesterol; LDLr, low density lipoprotein receptor; LFD, low-fat diet; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NTCP, Na⁺-taurocholate-cotransporting polypeptide; OPLS-DA, orthogonal projections to latent structures-discriminate analysis; PE, phosphatidylethanolamine(s); PC, phosphatidylcholine(s); PPAR α , peroxisome proliferator-activated receptor α ; PUFA, polyunsaturated fatty acid(s); SCD1, stearoyl-CoA desaturase-1; SM, sphingomyelin(s); SR-B1, scavenger receptor B1; TG, triacylglycerol(s); SHP, small heterodimer partner; TBA, total bile acid(s).

proteins involved in cholesterol metabolism [8]. More than 200 different phytosterols have been identified [9]; stigmasterol and β -sitosterol are the most commonly occurring ones. A daily intake of 2.0–2.5g phytosterols has been recommended for decreasing LDL-cholesterol levels by up to 10% and lowering the risk of cardiovascular diseases [10, 11]. A recent review also indicated that phytosterols are effective in lowering levels of plasma TG [12]. It was reported that phytosterol treatment reduced serum and hepatic TG levels by modulating the expression of lipid regulatory genes and *de novo* lipogenesis [13]. Phytosterols may also inhibit the absorption of fats by affecting bile acid homeostasis. Although the hypolipidemic effects of phytosterols as a group of compounds have been described, the effects of individual phytosterols on lipid metabolism and NAFLD have not been fully studied.

Different phytosterols may have different activities. In this study, we compared the effects of pure stigmasterol and β -sitosterol in alleviating a high-fat western-style diet (HFWD)-induced NAFLD in a mouse model. We hypothesized that phytosterols decrease the reabsorption of cholesterol and bile acid, which in turn affects lipid absorption and metabolism. Stigmasterol may be more effective than β -sitosterol in the actions because stigmasterol, but not β -sitosterol, has been reported to be an antagonist of farnesoid X receptor (FXR) [14], which may reduce circulating CM and suppress genes involved in hepatic lipid synthesis such as stearoyl-CoA desaturase-1 (SCD1) and fatty acid synthase (FAS) [15]. In this study, in addition to the several key markers for lipid metabolism and NAFLD, a lipidomic approach was taken to investigate the major lipid species that were altered by phytosterol treatments in liver and serum samples. The molecular mechanisms and pathways by which these two phytosterols alter lipid metabolism in the liver were also explored.

2. Materials and Methods

2.1. Animals, diet, and experimental design

Thirty-five 8-week old male C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were carried out under protocol 02–027, approved by the Institutional Animal Care and Use Committee at Rutgers University (Piscataway, NJ). Mice, five in each cage, were housed in plastic shoe-box cages in our animal facility under standard conditions (temperature 24–25°C, humidity 70–75% and lighting regimen of 12-hour light-dark cycle), with free access to food and water. The rodent diets were low-fat diet (LFD), HFWD, HFWD containing 0.4% stigmasterol (HFWD+stigma) and HFWD containing 0.4% β -sitosterol (HFWD+ β -sito) (prepared by Research Diets, Inc., New Brunswick, NJ). This HFWD was modified from the Western-style diet of Newmark et al [16] (which mimics dietary risk factors of western populations for colon cancer) by increasing the total fat content from 40% to 60% of total calories. The HFWD contained a higher level of fat (soybean oil at 34.6 g/kg and lard at 339.2 g/kg), lower levels of carbohydrates (maltodextran at 173.1 g/kg and sucrose at 95.3 g/kg) and lower amounts of calcium, vitamin D₃, choline, folate, and fiber than the normal AIN76A diet. The LFD was modified from the AIN76A diet. We previously used the HFWD to induce metabolic syndrome in mice and the compositions of these diets have been published [17]. Stigmasterol (95% pure) and β -sitosterol (95% pure) were obtained from Sigma-Aldrich (St.

Louis, MO). The level of 0.4% in the diet was based on the conclusion from the FDA that a daily dietary intake of 2g of phytosterols is needed to lower low-density lipoprotein cholesterol and reduce cardiovascular risk in humans [10]. Assuming a person consumes 500g of food in dry weight (2000 to 2500 kcal), based on caloric consumption [18] 2g/500g is equivalent to 0.4%.

After 1 week of acclimation on the chow diet, the mice were allocated into 4 groups and maintained on LFD (G1, n=5), HFWD (G2, n=10), HFWD+stigma (G3, n=10) and HFWD + β -sito (G4, n=10) for 17 weeks. Body weight of the mice, diet consumption and water consumption were measured weekly. Every two weeks, a 48-h fecal sample was collected from each cage. Fasting blood glucose level was measured every 4 weeks using an Ascensia Contour Blood Glucose meter (Bayer Healthcare LLC, Mishawaka, IN).

2.2. Sample collection and processing

To monitor the development of fatty liver, 100 μ l blood was collected from the tail vein after fasting (from 9:00 p.m. to 9:00 a.m.) every 2 or 4 weeks; serum lipid and alanine aminotransferase (ALT) levels were determined. After 17 weeks of treatment, the mice without fasting were euthanized by CO₂ asphyxiation. A blood sample was taken by cardiac puncture. Serum was prepared by centrifugation for 20 min at 3,000g, and then stored frozen at -80°C. Liver was quickly collected, weighed and half of the small lobe was cut and fixed in 10% buffered formalin for 24 h for histopathological analyses. Another piece of liver (~40mg) was stored in RNAlater solution (Ambion, NJ) for RNA extraction. The remaining liver tissue was quickly clamped with a Wollenberg clamp at liquid nitrogen temperature and stored at -80°C for lipidomic and bile acid (BA) analyses. Gallbladder was collected and stored at -80°C for BA analyses. Ileum was cut, washed with ice-cold saline for RNA extraction. The whole small intestine was stored frozen at -80°C for BA analyses. Interscapular brown adipose tissue and visceral white adipose tissues (mesenteric, epididymal, and retroperitoneal depots) were collected, weighed and stored.

2.3. Biochemical analysis of serum and liver samples

Serum levels of ALT were determined using ALT Discrete Pak kit (Catachem Inc., Bridgeport, CT). Serum lipid levels, total cholesterol (TC) levels, TG levels, and high-density lipoprotein cholesterol (HDL-C) levels were determined using commercial kits according to manufacturer's instruction (Pointe Scientific, Inc. Canton, MI). Non-high-density lipoprotein cholesterol (non-HDL-C) levels were calculated according to the following equation:

$$\text{non-HDL-C} = \text{TC} - \text{HDL-C}$$

Lipid was extracted from liver and fecal samples according to the method described by Manley *et al.* [19]; TG and cholesterol levels were measured as described above.

2.4. Histological analysis of liver

The formalin-fixed liver tissue samples were embedded in paraffin, sectioned serially at 4- μ m thickness and stained with hematoxylin and eosin. Lesions were evaluated by a

histological scoring system described by Liang *et al.* [20]. Macrovesicular steatosis, microvesicular steatosis and hepatocellular hypertrophy were separately scored and the severity in each category was graded, based on the percentage of the total area affected, into the following categories: 0 (<5%), 1 (5–33%), 2 (34–66%) and 3 (>66%). Inflammation was evaluated by counting the number of inflammatory foci per field at a 100 x magnification. Five different fields were counted and the average was subsequently scored into the following categories: normal (<0.5 foci), slight (0.5–1.0 foci), moderate (1.0–2.0 foci), severe (>2.0 foci).

2.5. Determination of bile acid pool size

BA was extracted from liver, gallbladder, small intestine and fecal samples according to the method of Kim *et al.* [21] with minor modifications. For liver and feces, about 100 mg samples were homogenized in 0.5 ml PBS, and bile acids were extracted twice with 1.5ml ethanol, for 1h each time. After centrifugation at 4000g, the supernatant was collected for BA assay. Whole intestine with contents was homogenized in 3ml PBS, and the homogenizer was washed with 3ml ethanol. To the combined mixture, 6ml ethanol was added and the mixture was put on a shaker for 1h to extract BA. After centrifugation, the BA in the precipitate was extracted again with 6ml ethanol for 1h. The combined supernatant was collected and diluted 5 times for BA assay. For the gallbladder sample, 1ml PBS solution was added. After breaking the gallbladder using a pipette tip and vortexing, the solution was diluted 5 times for BA assay. For BA assay, a Total Bile Acid Assay kit (Diazyme Laboratories, Poway, CA) was used.

2.6. Lipidomic analysis of liver and serum samples

Frozen liver tissue samples (~30mg) were weighed and then pulverized in a CryoMill machine (Retsch, Germany) with a stainless ball at liquid nitrogen temperature. To the pulverized tissue powder, 1ml of 0.1M HCl in 50:50 methanol:H₂O was added; the mixture was vortexed and then placed in a –20°C freezer for 30 min. Then 0.5ml of chloroform was added and vortexed to mix well, and the mixture was placed on ice for 10min. Samples were centrifuged at 15,000 RCF for 10min and the chloroform phase at the bottom was transferred to a glass vial using a Hamilton syringe. For a second extraction, chloroform (0.5ml) was added to the remaining material and the extraction was repeated. For serum sample, 50µl serum was extracted with 500µl of ethyl acetate three times. The combined extracts were dried under a stream of nitrogen and re-dissolved in a mixture of methanol: chloroform: 2-propanol (1: 1: 1) using a ratio of 1ml of solvent per 25mg of initial tissue weight.

Free fatty acids and lipids were analyzed on an Agilent 6550 iFunnel Q-TOF mass spectrometer coupled to a 1290 Infinity UHPLC system. Each sample was analyzed twice using the same LC gradient but different ionization modes on mass spectrometer to cover both the positive charged and negative charged species. The LC separation was achieved on an Agilent Poroshell 120 EC-C18 column (150×2.1mm, 2.7µm particle size) at a flow rate of 150µl/min. The gradient was generated with solvents A and B as follows: 0min, 25% B; 2min, 25% B; 4min, 65% B; 16 min, 100 %B; 20min, 100% B; 21min, 25% B; 27min, 25%

B. Solvent A was 1mM ammonium acetate + 0.2% acetic acid in water: methanol (90:10). Solvent B is 1mM ammonium acetate + 0.2% acetic acid in methanol: 2-propanol (2:98).

The lipid name, sample name, and normalized peak area were fed to the SIMCA14 software package (Umetrics, Umea, Sweden) for orthogonal projections to latent structures-discriminate analysis (OPLS-DA). To refine the analysis, variable importance projection (VIP) of each lipid was measured. VIP values exceeding 1.0 and $p < 0.05$ in one-way ANOVA were selected as changed metabolites. Commercial databases, metaboanalyst 3.0 (<http://www.metaboanalyst.ca>), were used for qualitative analysis and searching for metabolites in the lipid biosynthesis pathways [22].

2.7. Total RNA extraction, reverse transcription and real-time q-PCR analysis

Total RNA was extracted from liver samples using RNeasy mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocol. Quality of RNA was determined by measuring absorbance at 260 and 280 nm. Reverse transcription reaction was done using a Superscript kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol with a GeneAmp PCR system 9700 (Applied Biosystems; Foster City, CA). The primer sequences of hydroxymethylglutaryl coenzyme A reductase (HMGCoAR), low density lipoprotein receptor (LDLr), scavenger receptor B1 (SR-B1), ATP binding cassette transporter A1 (ABCA1), ATP binding cassette transporter 5 (ABCG5), SCD1, FAS, peroxisome proliferator-activated receptor α (PPAR α), cluster of differentiation 36 (CD36), small heterodimer partner (SHP), cholesterol 7 α -hydroxylase gene (CYP7A1), sterol 12 α -hydroxylase (CYP8B1), sterol 27-hydroxylase (CYP27A1), bile salt export pump (BSEP), Na⁺-taurocholate-cotransporting polypeptide (NTCP), ileal apical sodiumdependent bile acid transporter (ASBT) and fibroblast growth factor 15 (FGF15) are listed in Supplementary Table S1. Real time quantitative PCR was performed using a Power SYBR® Green PCR Master Mix kit (ABI Co., Ltd. USA) with an ABI ViiATM 7 system. The mRNA expression levels were calculated as the ratio of optical density to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amount of the target gene expression was calculated by the 2^{-CT} method.

2.8. Statistical analysis

The normality of data was verified with the Shapiro-Wilk test using SPSS 19.0 software package (IBM, USA) and $p > 0.05$ was set as the data meet the assumption. Data were presented as mean $\pm$ standard deviation (except in Fig. 6 as mean $\pm$ standard error) and analyzed using one-way ANOVA followed by Duncan's new multiple range test among multiple groups. In special situations, student's t-test was used to determine the difference between two groups. A significance level $p < 0.05$ was set for all the tests.

3. Results

3.1. Effects of dietary phytosterols on body weight, food intake, fatty tissue weights and liver weights

As expected, the growth rate of the LFD group (G1) was slower than the three groups on HFWD. No significant difference in body weight among these three groups was observed in

the first few weeks of the experiment (Fig. 1a). However, after 8 weeks, the body weights of the stigmasterol treatment group (G3) were significantly lower than those in the HFWD control group (G2). β -Sitosterol treatment (G4) slightly decreased the body weights compared to G2, but the changes were not statistically significant. There was no significant difference in food intake among the groups (Fig. 1b).

At week 17 of the experiment, the mice were sacrificed; adipose tissues and liver were collected and weighed (Table 1). The total adipose tissue weight was significantly higher in G2 than G1 (8.34 ± 0.77 vs. 3.43 ± 0.95 g/100g BW). Both phytosterol treatments significantly increased total adipose tissue weight compared to G2, which is mainly due to the increase in epididymal white adipose tissue. Interestingly, stigmasterol (G3) significantly lowered the liver weight (2.08 ± 0.42 g vs. 2.60 ± 0.61 g in G2), but β -sitosterol did not (no significant difference between G4 and G2).

3.2. Liver histology

In order to examine the protective effects of stigmasterol and β -sitosterol against fatty liver development, histological analysis of liver tissue from each mouse was conducted after H&E staining. The liver from LFD mice (G1) were histologically normal (Fig. 1c G1). HFWD induced fatty liver with large areas of microvesicular steatosis, regions of evolving macrovesicular fatty change, and hepatocellular hypertrophy in the liver tissue (Fig. 1c G2). Stigmasterol treatment decreased the macrovesicular steatosis, microvesicular steatosis and hepatocellular hypertrophy (compared to G2), with more normal areas and even almost normal histology in some samples (Fig. 1c G3). As compared to G2, the histological score was significantly lower in G3 ($p < 0.05$), indicating that stigmasterol significantly alleviated HFWD-induced NAFLD. However, β -sitosterol was less effective in the protective function (Fig. 1c G4, 1d).

3.3. Effects on serum lipid, fasting blood glucose and serum ALT levels

Serum total cholesterol, HDL-C, non-HDL-C and TG concentrations were determined at different time points during the experiment (Fig. 2). Treatment with HFWD (G2) caused significant increases in TC, non-HDL-C, HDL-C and TG (only in week 10) levels compared to G1, confirming the hyperlipidemic effect of HFWD. For mice fed HFWD, stigmasterol or β -sitosterol treatment significantly decreased the total cholesterol and non-HDL-C concentrations (Fig. 2a, b), but only stigmasterol treatment decreased HDL-C level in week 8 (Fig. 2c). TC: HDL ratio was increased from 2.3:1 (G2) to 3.3:1 (G3) in week 8. There were no significant differences in TG levels among different groups (Fig. 2d). The fasting blood glucose level was significantly increased in mice treated with HFWD, and it was not significantly altered by either phytosterol (data not shown). The serum ALT levels were higher in G2 than G1 on week 10 and beyond, suggesting the induction of liver damage by HFWD. Stigmasterol was effective in preventing the elevation of ALT levels, but β -sitosterol was not effective (Fig. 2e).

3.4. Effects on bile acid levels

To further investigate the effects of phytosterol treatments on lipid metabolism, their effects on BA levels were measured. Treatment with HFWD significantly increased the amounts of

total bile acid (TBA) and intestinal TBA, and these increases were prevented by phytosterol treatments. However, the dietary treatment did not significantly change the TBA levels in the liver and gallbladder (Fig. 2f). β -Sitosterol significantly, but stigmasterol nonsignificantly, increased the fecal excretion BA.

3.5. Effects on hepatic lipid and fecal lipid levels

Mice in G2 had significantly higher levels of hepatic total lipids, TG, TC and fecal lipid levels than mice in G1 (Table.1). With stigmasterol treatment (G3), the hepatic levels of total lipids, cholesterol and TG were significantly decreased, while fecal lipid and cholesterol levels were markedly increased (compared to G2). The effects of β -sitosterol were similar but to a less extent. These results suggest that both phytosterols are effective in lowering hepatic lipid levels through increasing their fecal excretion.

3.6. Lipidomics analysis of liver samples

A total of 159 individual lipids were significantly altered by HFWD (G2 vs. G1). In comparison to G2, stigmasterol treatment (G3) significantly altered 63 species in liver including DG, PE, SM, TG, PC and CM (G3 vs. G2), but β -sitosterol treatment (G4) only significantly changed 7 species (Supplementary Table S2).

The changes in the levels of the more abundant species of each lipid class are depicted in Fig. 3. Among the 17 DG species analyzed, most of the species were either increased or not affected by HFWD. Of the 10 more abundant DG species (Fig. 3a), most species were increased by HFWD; stigmasterol (but not β -sitosterol) partially, but significantly, prevented the HFWD-induced increase in DG (36:3, 36:2, 36:1). Otherwise, there was no clear pattern of effects. Among the 48 TG species analyzed, most species were increased by HFWD, but those with short fatty acid chains and fewer double bonds (up to TG 50:3), presumably through de novo biosynthesis, were decreased by HFWD. Of the 16 more abundant TG species (Fig. 3d), some were increased and others decreased by HFWD, and stigmasterol was more effective than β -sitosterol in preventing these changes. For example, stigmasterol (but not β -sitosterol) significantly increased TG (52:4, 52:3, 52:2, 56:8) and decreased TG (50:3, 50:5), in both cases neutralizing the HFWD-induced effects.

Of the 6 more abundant CM species (Fig. 3b), stigmasterol increased the rather abundant CM (d18:1/22:0, d18:1/24:3) to the highest level among groups. For other species, stigmasterol and β -sitosterol did not prevent the decrease caused by HFWD. Of the 5 more abundant SM species (Fig. 3c), stigmasterol (but not β -sitosterol) prevented the HFWD-induced decrease in SM (d18:1/22:0, d18:1/24:3).

For the 12 PE species analyzed, treatment with HFWD slightly decreased most species and the total quantity of PE. Of the 5 more abundant species (Fig. 3e), stigmasterol treatment was effective in preventing the decrease (PE 38:4, 38:6 and 38:3) caused by HFWD; β -sitosterol treatment was less effective. Among the 37 PC species analyzed, more species were decreased (than increased) by the low-choline content HFWD. Of the 15 more abundant species (Fig. 3f), stigmasterol and β -sitosterol had little effect. Stigmasterol (but not β -sitosterol) only prevented the HFWD-induced increase in the levels of PC (38:3, 36:3).

Among the 33 FFA analyzed, only 1 species was increased by HFWD, and of the 10 more abundant species (Fig. 3g), both phytosterols had little effect.

To discriminate the treatment effects on different lipid species, a principal component analysis (PCA) score plot was employed to visualize the sample distribution of the multivariate patterns. A plot of the first three principal factors of variability for all lipid species in liver samples showed that clusters of lipid species were significantly changed by HFWD and stigmasterol (Fig. 4a). The global profiles among G1, G2 and G3 were highly distinct. The first principal component factor efficiently separated the G3 samples from G2 samples and located the profile of G3 between G1 and G2. The second and third principal components could also largely segregate G1, G2 and G3. These three principal component factors together explain more than 85% of the variance. However, β -sitosterol treatment (G4) samples were not significantly distinct from G2 samples. PCA was conducted to investigate the differences of DG in different groups (Fig. 4b). The first principal component factor efficiently separated the samples of G3 from G2, and made G3 indistinguishable from G1, while the third principal component factor efficiently separated the G2 and G3 samples from G1. Similar results were observed in the analysis of TG (Fig. 4c). The results demonstrated that stigmasterol treatment effectively prevented the HFWD-induced changes in total hepatic lipids, DG and TG.

On the other hand, the PCA of FFA showed that the global profiles among samples from different groups were not distinct (Fig. 4d). This is consistent with the results shown in Fig. 3g that the levels of hepatic FFAs were not significantly altered by the HFWD or the phytosterol treatments.

3.7. Pathway analysis

The OPLS-DA showed that mice in G3 and G4 were well separated from G2 (Fig. 5a, c). The lipid species with VIP > 2.0 in the S-plot, namely SM (d18:1/22:0), TG (52:2, 52:4, 52:1, 50:5 and 56:8), PC (38:3 and 36:3) and DG (36:3 and 36:2) (Fig. 5b) together with other 38 species with VIP > 1.0 were screened out as potential markers for stigmasterol. PC (38:3, 30:0, 36:3 and 36:2), DG (40:5 and 40:4) and TG (54:8, 52:7, 50:5 and 52:5) (Fig. 5d) together with other 34 species with VIP > 1 were potential markers for β -sitosterol. Pathway analysis showed that these metabolites are in the pathways of glycerophospholipid metabolism, alpha-linolenic acid metabolism and linoleic acid metabolism (Fig. 5e, f). A summary of the parameters obtained in the pathway analysis are shown in Supplementary Table S3.

3.8. Analysis of gene expression changes with q-PCR

To explore the mechanisms of the above observed phytosterol actions in decreasing hepatic lipid accumulation and bile acid pool size, we analyzed the expression of genes involved in lipid and bile acid metabolism with q-PCR (Fig. 6). In the liver, the mRNA expression of HMGCoAR, was significantly decreased by HFWD, but the decrease was prevented by both phytosterols and further induced by stigmasterol (Fig. 6a). Similar patterns of changes were observed in the expression of LDLr, but significant differences were only seen with β -sitosterol. β -Sitosterol treatment also significantly increased the expression of ABCG5. The

de novo lipogenesis related genes, such as SCD1 and FAS, were significantly suppressed by stigmasterol, but not β -sitosterol. Both phytosterols significantly increased the expression of PPAR α and decreased the expression of CD36.

The hepatic expression of CYP7A1 was suppressed by HFWD, but increased by treatment with β -sitosterol (Fig. 6b). Stigmasterol significantly increased the expression of CYP8B1 and CYP27A1, while the effect of β -sitosterol was not statistically significant. The expression of BSEP and NTCP, which are involved in bile acid excretion to gallbladder and uptake of bile acid from blood, respectively [23], was decreased by HFWD but not significantly altered by phytosterol treatments. Both phytosterols significantly increased the hepatic expression of FXR, while only β -sitosterol decreased the expression of SHP. In ileum samples (Fig. 6c), significant effects of either phytosterol were not observed in the expression of FXR, SHP, ASBT and FGF15, possibly due to the large standard deviations.

3.9. Lipidomics analysis of serum samples

To investigate the alterations in the levels of lipid species by phytosterols, lipidomic analysis of serum samples was conducted and the results were compared by ANOVA. In serum samples, a total of 98 individual lipids were altered in quantity by HFWD. Stigmasterol treatment significantly changed 39 species in serum including DG, PE, SM, TG, PC and CM, while β -sitosterol treatment significantly changed 54 species (Supplementary Table S4).

The changes in the more abundant species of each class in serum are depicted in Fig. 7. Among the 13 CM species analyzed, most species were increased by HFWD and decreased by stigmasterol, but no pattern of change was observed with β -sitosterol. Of the 7 more abundant species (Fig. 7a), stigmasterol (but not β -sitosterol) treatment decreased the levels of CM d18:1/ (26:4, 24:1, 24:0 and 22:3).

Different from the lipids in liver, both stigmasterol and β -sitosterol treatments had little effect in affecting the serum TG (Fig. 7b) and DG levels (Supplementary Table S4). Most of serum FFA were not significantly affected by stigmasterol and β -sitosterol, except for circulation PUFA (C20:4, 20:3), whose elevation by HFWD was prevented by both phytosterols (Fig. 7c). Similar to liver samples, the levels of many PEs were decreased by HFWD and some were prevented by β -sitosterol, and to less extent by stigmasterol (Fig. 7d). Of the more abundant SM and sphingosines species (Fig. 7e), stigmasterol significantly increased SM (d18:1 22:0) and decreased sphingosines (24:0 and 24:1) levels. Of the 11 more abundant PCs, β -sitosterol significantly increased levels of most PC (34:1, 36:4, 38:4, 38:6, 38:5 and 36:1), but stigmasterol only affected a few species (Fig. 7f).

PCA showed that serum lipids were significantly changed by dietary treatments, and due to stigmasterol treatment, clusters of G3 were separated from that of G2, (Supplementary Fig.S1). OPLS-DA separated both G3 and G4 from G2 (Supplementary Fig. S2 a-d). Pathway analysis showed the potential biomarkers that were changed by the two phytosterols (Supplementary Fig. 2 e,f). The results are generally in agreement with the results in Fig. 7.

4. Discussion

Although the cholesterol-lowering properties of phytosterols have been well described, little is known about the effects of specific phytosterols on lipid metabolism and NAFLD. Our data clearly showed that supplementation of stigmasterol to HFWD significantly reduced the body weight gain, serum cholesterol, histological score and ALT levels. The present study demonstrated that stigmasterol is more effective than β -sitosterol in decreasing hepatic lipid accumulation and NAFLD in mice fed a HFWD.

The hypocholesterolemic effects of phytosterols have been studied in serum [11]. The present study highlights the hepatic cholesterol lowering effects (about 50% decreased compared to HFWD group) of both phytosterols (Table 1). Stigmasterol and β -sitosterol increased hepatic HMGCoAR mRNA expression, the rate-limiting enzyme in cholesterol biosynthesis [24], possibly due to the lowering hepatic cholesterol levels by these phytosterols. Therefore, the cholesterol lowering effects are mainly due to decreased reabsorption of cholesterol as reflected in its higher fecal contents (Table 1). It has been reported that hepatic accumulation of free cholesterol is a contributing factor to the pathogenesis of NAFLD, through the activation of Kupffer cells, stellate cells and hepatocytes [3]. The presently observed hepatic cholesterol lowering effect of phytosterols should contribute to the alleviation of NAFLD.

With the lower hepatic levels of cholesterol in the phytosterol-treated mice, the substrate level for BA biosynthesis would be lower. The lower intestinal levels and higher fecal contents of BA (Table 1) suggest that phytosterols play an important role in decreasing BA reabsorption. Our results are consistent with a previous report that bile acid excretion was significantly increased with phytosterol treatment [25]. About 90~95% of BA excreted in the bile return to the liver through ileal reabsorption, which is mediated by ASBT [26]. In this study, the expression of ASBT was not significantly changed by phytosterols. Whether phytosterols inhibit the activity of ASBT remains to be determined. BA are released into the small intestine after meal ingestion and facilitate intestinal absorption of dietary fat. The elevated mRNA levels of enzymes in BA synthesis – CYP7A1, CYP81 or CYP27A1, key enzymes in the bile acid biosynthesis pathway [27], was found with both phytosterols. This is probably a compensatory response to the lower substrate levels of BA. The lower intestinal BA levels would decrease the absorption of lipids from the diet, leading to the attenuation of body weight gain and lipid accumulation in liver. In addition, the lowered expression of CD36 caused by phytosterols (Fig. 6) may decrease hepatic uptake of fatty acid from the blood [28].

Lipidomics is a sensitive and effective approach for detecting biological responses by investigating the individual lipid species. Analysis of lipidomic changes in serum or liver could provide valuable information in study NAFLD [29–31]. The HFWD generally increased the hepatic levels of DG and TG, but decreased those of SM, PE and PC. Stigmasterol was more effective than β -sitosterol in preventing some of these changes (Fig. 3 and Table 1). The results of OPLS-DA and pathway analysis (Fig. 5) are consistent with the conclusion and ascertained the changes in some of the key lipid species. The increase in DG and TG is consistent with a similar observation in human NAFLD steatotic livers [32]

and stigmasterol effectively prevented most of these changes. In our study, TG with highly polyunsaturated fatty acids (PUFA), derived from dietary sources, was significantly elevated by the HFWD and the elevation was prevented by stigmasterol. The total hepatic TG content was significantly decreased by stigmasterol treatment (Table 1). SM and CM are two major class of sphingolipids that regulates cell cycle progression, growth and cellular metabolism [33]. It is interesting that stigmasterol increased the CM levels in the liver, but decreased CM levels in the serum. A decrease of serum CM was associated with reduced hepatic fat accumulation and improved insulin sensitivity [34–38]. Such actions could contribute to the lipid-lowering effects of stigmasterol observed herein.

Accumulation of FFA in the liver is believed to be a major cause of cell injury in NAFLD [7]. However, hepatic FFA levels were not significantly affected by HFWD in our study. The results are consistent with a previous report that hepatic FFA contents remained unaltered in NAFLD [29]. Although both phytosterols did not change the levels of major constituents of circulating FFAs, they decreased circulating PUFA, which may undergo lipid peroxidation and generate toxicity. We also found both phytosterols significantly increased the expression of PPAR α , which may increase the β -oxidation of hepatic fatty acid [39]. It is interesting that stigmasterol was effective in decreasing hepatic lipid accumulation while increasing the weight of epididymal white adipose tissue. Whether this is associated with ectopic lipid deposition [40–43], needs to be investigated. On the other hand, the effects of β -sitosterol on these parameters are not as clear. It was reported previously that intake of phytosterol protected against BW gain [44, 45]. This effect is associated with increase in fecal fat loss. In this study, PS treatment caused an increase on total adipose tissue weight. The loss of BW is probably due to loss of water or muscle.

The more potent preventing NAFLD effects of stigmasterol (than β -sitosterol) may be related to its effects in lowering the expression of lipogenic genes, FAS and SCD1 (Fig. 3) and decreasing circulating CM levels (Fig. 7a) [46]. Stigmasterol (but not β -sitosterol) has been reported to be an antagonist of FXR [14]. It was reported that inhibition of intestinal FXR would lower CM synthesis, resulting in lower serum and ileum CM levels [46], and lowered levels of circulating CMs could decrease hepatic TG accumulation via down regulation of hepatic SREBP1C and lipogenesis [46]. Our results showed that stigmasterol decreased hepatic TG accumulation, but did not suppress the expression of FXR, SHP, FGF15 in ileum samples. The interaction of stigmasterol with FXR in our experimental system remains to be further studied.

In conclusion, stigmasterol and β -sitosterol, at a dose comparable to that suggested for humans by the FDA, are shown to decrease the reabsorption or absorption of cholesterol, bile acids and dietary lipids, leading to the attenuation of body weight gain, hepatic lipid accumulation and alleviation of NAFLD. Stigmasterol is more effective than β -sitosterol in these actions, possibly through its decreasing the expression of some lipogenic genes and lowering circulating CM levels. The results suggest that consumption of phytosterols has activities in preventing or alleviating diet-induced NAFLD. Phytosterols are typically consumed by humans as a mixture of different forms in plant-based foods. A phytosterol mixture with a high stigmasterol to β -sitosterol ratio may provide added advantage as a

dietary supplement. Further studies are needed to determine the potential use of phytosterols as preventive and adjuvant therapeutic agents for NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Stigmasterol and β -sitosterol alleviate nonalcoholic fatty liver disease in mice
- Mechanism of action – reducing bile acid reabsorption and lipid absorption
- Stigmasterol is more effective than β -sitosterol in these actions
- Stigmasterol also decreases circulating ceramide levels and hepatic lipogenesis

Figure 1.

Effects of stigmasterol and β -sitosterol on body weight (a), dietary consumption (b) and liver histology (c and d). The mice were maintained on experimental diets as described in Materials and Methods and were sacrificed on week 17. The data are shown as mean $\pm$ SD (n=5 for G1 and n=10 for G2–4). ** indicates statistical difference from the HFWD group (p < 0.01, t-test). ^{a,b,c} Designate difference among groups (p < 0.05, ANOVA).

Figure 2.

Serum lipid and ALT levels and total bile acid levels. a, total cholesterol concentrations; b, non - HDL - cholesterol concentrations; c, HDL - cholesterol concentrations; d, triacylglycerols concentrations; e, ALT levels; f, bile acid levels. The data are presented as mean $\pm$ SD. ^{a,b,c} Designate difference among groups ($p < 0.05$, ANOVA). Abbreviations: ALT, alanine aminotransferase; TBA, total bile acids

Figure 3.

Serum lipid profiles analyzed by lipidomics. The designation of each panel is indicated in the figure. Peak area is presented as mean $\pm$ standard deviation. (n=4 for G1 and n=5 for G2–4). ^{a,b,c}Designate difference among groups ($p < 0.05$, ANOVA). Abbreviations: DG, diacylglycerol(s); CM, ceramides; TG, triacylglycerol(s); PE, phosphatidylethanolamine(s); PC, phosphatidylcholine(s); FFA, free fatty acid(s)

Figure 4.

Principal component analysis (PCA) of lipid species in liver samples. a. PCA of all the hepatic lipids that were significantly changed by stigmasterol or β -sitosterol treatment in lipidomic study; b. PCA of all the hepatic DG detected; c. PCA of all the hepatic TG detected; d. PCA of all the hepatic FFAs detected. One spot represents an individual animal (n=4 for G1 and n=5 for G2–4). Principal component analysis (PCA) were conducted with SPSS 19.0 software package (IBM, USA). Abbreviations: DG, diacylglycerol(s); TG, triacylglycerol(s); FFA, free fatty acid(s)

Figure 5.

OPLS-DA (a, b, c and d) and pathway analysis (e and f) based on data from lipidomics analysis of liver samples. a. In the score plot, mice from HFWD and stigmasterol treatment group were well separated; b. using a statistically significant threshold of variable confidence approximately 0.6 and VIP value more than 2.0 in the S-plot, a number of lipid species were screened out as potential markers. c. In the score plot, mice from HFWD and stigmasterol treatment group were well separated; d. using a statistically significant threshold of variable confidence approximately 0.6 and VIP value more than 2.0 in the S-plot, a number of lipid species were screened out as potential markers. e. Possible disturbed metabolic pathways of

the potential biomarkers for stigmasterol and β -sitosterol treatment. 1.Glycerophospholipid metabolism, 2.Linoleic acid metabolism,3.alpha-Linolenic acid metabolism, 4.Glycosylphosphatidylinositol(GPI)-anchor biosynthesis, 5.Glycerolipid metabolism, 6.Sphingolipid metabolism, 7.Arachidonic acid metabolism. f. Pathway of select glycerophospholipid metabolites from the KEGG database with indications of quantified lipid classes. Red and green colored boxes indicate increased and decreased lipid classes by stigmasterol treatment; yellow boxes indicate some spieces of lipid class was increased but others decreasedby stigmasterol treatment, white boxes indicate no change of lipid classesby stigmasterol treatment. Abbreviations: DG, diacylglycerol(s); CM, ceramides; TG, triacylglycerol(s); PE, phosphatidylethanolamine(s); PC, phosphatidylcholine(s); SM, sphingomyelin(s)

Figure 6.

mRNA expression of hepatic lipid metabolism genes (a), hepatic bile acid metabolism genes (b) and intestinal bile acid metabolism genes (c). mRNA expression was determined by qPCR in samples of week 17 as described in Materials and Methods. Data are expressed as mean $\pm$ standard error (n=5). * indicates significant difference from G2 ($p < 0.05$, t-test). Abbreviations: HMGCoAR, hydroxymethylglutaryl coenzyme A reductase; LDLr, low density lipoprotein receptor; SR-BI, scavenger receptor B1; ABCA1, ATP binding cassette transporter A1; ABCG5, ATP binding cassette transporter 5; SCD1, stearoyl-CoA desaturase-1; FAS, fatty acid synthase; PPAR α , peroxisome proliferator-activated receptor α ; CD36, cluster of differentiation 36; FXR, farnesoid X receptor; SHP, small heterodimer partner; CYP7A1, cholesterol 7 α -hydroxylase; CYP8B1, sterol 12 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; BSEP, bile salt export pump; NTCP, Na⁺-taurocholate-cotransporting polypeptide; ASBT, ileal apical sodium-dependent bile acid transporter; and FGF15, fibroblast growth factor 15.

Figure 7.

Hepatic lipid profiles analyzed by lipidomics. The designation of each panel is indicated in the figure. Peak area is presented as mean $\pm$ standard deviation. (n= for G1 and n=5 for G2–4). a,b,c Designate difference among groups ($p < 0.05$, ANOVA). Abbreviations: CM, ceramide(s); FFA, free fatty acids; TG, triacylglycerol(s); PE, phosphatidylethanolamine(s); SM, sphingomyelin(s); PC, phosphatidylcholine(s)

Table 1.

Fat weights and lipid contents in liver and feces *.

	G ₁ LFD	G ₂ HFWD	G ₃ HFWD +stigma	G ₄ HFWD +β-sito
Total adipose tissue [†]	3.43±0.95 ^c	8.34±0.77 ^b	9.34±1.30 ^a	9.45±1.26 ^a
Brown adipose tissue [†]	0.59±0.09 ^b	1.15±0.20 ^a	1.08±0.26 ^a	1.10±0.21 ^a
Total white adipose tissue [†]	2.82±0.94 ^c	7.20±0.90 ^b	8.60±1.16 ^a	8.23±1.36 ^a
mesenteric white adipose tissue [†]	0.62±0.26 ^b	2.78±0.60 ^a	2.43±0.60 ^{a#}	2.66±0.67 ^a
epididymal white adipose tissue [†]	1.73±0.54 ^c	3.36±1.01 ^{bc}	4.96±1.41 ^a	4.37±1.57 ^{ab}
retroperitoneal white adipose tissue [†]	0.47±0.17 ^b	1.06±0.16 ^a	1.21±0.20 ^a	1.12±0.28 ^a
Liver weight(g)	1.24±0.38 ^c	2.60±0.61 ^a	2.08±0.42 ^{abc#}	2.33±0.89 ^{ab}
Hepatic lipids (mg/100mg liver)	14.18±3.82 ^c	26.13±2.76 ^a	20.22±3.65 ^{bc}	22.68±5.66 ^{ab}
Hepatic cholesterol (mg/g liver)	2.53±0.57 ^b	5.19±1.47 ^a	2.65±0.26 ^b	2.41±0.66 ^b
Hepatic triacylglycerols (mg/g liver)	2.88±0.98 ^c	28.55±3.99 ^a	17.36±8.46 ^b	21.87±7.86 ^{ab}
Fecal lipids (mg/g feces)	26.12±2.49 ^d	58.05±3.65 ^c	106.86±6.44 ^a	97.59±0.82 ^b
Fecal cholesterol(mg/g feces) [#]	2.75±0.84 ^c	2.64±0.70 ^d	35.70±2.47 ^a	26.19±2.26 ^b
Fecal triacylglycerols(mg/g feces) [#]	1.12±0.17 ^b	2.27±0.72 ^a	1.45±0.16 ^b	1.44±0.23 ^b

* Data are shown as mean value ± standard deviation, n=5 for G₁ and n=10 for G₂, G₃ and G₄, unless otherwise indicated.

^a Mean values within a row with different letters are significantly different ($p < 0.05$, ANOVA).

^b Mean values within a row with different letters are significantly different ($p < 0.05$, ANOVA).

^c Mean values within a row with different letters are significantly different ($p < 0.05$, ANOVA).

^d Mean values within a row with different letters are significantly different ($p < 0.05$, ANOVA).

[†] Unit: g/100g BW.

[#] Significant difference from G₂ ($p < 0.05$, t-test).

[#] n=4 for G₁, G₂, G₃ and G₄ from week 16 in fecal lipids analysis.