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Glycosidic Bond Cleavage is Not Required for Phytosteryl Glycoside-Induced Reduction of Cholesterol Absorption in Mice

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Abstract

Phytosteryl glycosides occur in natural foods but little is known about their metabolism and bioactivity. Purified acylated sterol glycosides (ASG) were compared with phytosteryl esters (PSE) in mice. Animals on a phytosterol-free diet received ASG or PSE by gavage in purified soybean oil along with tracers cholesterol- d_7 and sitostanol- d_4 . In a three-day fecal recovery study, ASG reduced cholesterol absorption efficiency by $45 \pm 6\%$ compared with $40 \pm 6\%$ observed with PSE. Four hours after gavage, plasma and liver cholesterol- d_7 levels were reduced 86% or more when ASG was present. Liver total phytosterols were unchanged after ASG administration but were significantly increased after PSE. After ASG treatment both ASG and deacylated sterol glycosides (SG) were found in the gut mucosa and lumen. ASG was quantitatively recovered from stool samples as SG. These results demonstrate that ASG reduces cholesterol absorption in mice as efficiently as PSE while having little systemic absorption itself. Cleavage of the glycosidic linkage is not required for biological activity of ASG. Phytosteryl glycosides should be included in measurements of bioactive phytosterols.

Keywords

Stable isotopes; Plant sterols; Mass spectrometry; Lipid absorption

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Conflict of interest Dr. Ostlund and Washington University have a financial interest in Lifeline Technologies, a biotechnology company developing bioactive phytosterols. Lifeline products were not used in this study, and Lifeline and the authors have no financial interest in phytosteryl glycosides.

Introduction

Phytosterols are structurally similar to cholesterol, occur naturally in plant-derived foods, and reduce cholesterol absorption [1-4] and plasma LDL-cholesterol [5, 6]. Because of these potentially beneficial health effects, the US National Cholesterol Education Program Adult Treatment Panel III has recommended adding 2.0 g/day phytosterols to the diet of adults to reduce LDL-cholesterol and coronary heart disease risk [7, 8].

To reach the 2.0 g/day of phytosterols, pure phytosterols (free or esters) have been routinely added to various functional foods. The chemical or physical form of the phytosterol is potentially important for bioactivity because crystalline phytosterols are not readily soluble in bile and do not reduce cholesterol absorption [9]. When properly formulated with lecithin or other emulsifiers, free phytosterols do reduce cholesterol absorption and plasma LDL cholesterol [10]. Phytosteryl esters solubilized in the tri-glyceride phase of margarines are also bioactive [11, 12].

Phytosterols are conjugated to various components in food matrices and not all have been tested for bioactivity [13]. Recently, we have demonstrated that pure phytosteryl esters supplemented at an amount achievable from a diet (459 mg phytosterols/day) reduce cholesterol absorption and increase fecal cholesterol excretion in humans [14]. Furthermore, phytosterols present in natural food matrices (449 mg/day) are also bioactive in reducing cholesterol absorption and enhancing fecal cholesterol excretion [15]. Because phytosteryl glycosides comprise a significant proportion of naturally occurring phytosterols in some foods [16-18], we have studied them and demonstrated that phytosteryl glycosides (mostly acylated sterol glycosides, ASG) purified from soybean-derived lecithin reduce cholesterol absorption efficiency in humans [19]. However, several questions remain unanswered regarding their mechanisms of action. Phytosteryl glycosides could act intact, as glycosides (with the fatty acid cleaved), or as free phytosterols (with both fatty acid and glucose cleaved).

ASG consist of a fatty acid and a glucose moiety in addition to the phytosterol [19]. Previous work has shown that fatty acids are cleaved from glycosylated phytosterols in vitro by pancreatin and cholesterol esterase but the sugar moiety itself is not removed [20]. Acetylated ^{14}C -sitosteryl glucoside was not absorbed in the stomach or small bowel of rats after intragastric administration [21]. However, the ^{14}C label was located in the phytosterol moiety, which did not enable the authors to answer whether or not any cleavage had occurred in vivo.

In this study we prepared purified ASG and tested the hypotheses that ASG is active in reducing cholesterol absorption efficiency in mice and that it is cleaved to SG or free phytosterols in mice consuming a cholesterol and phytosterol-free diet.

Materials and Methods

Materials

[25,26,26,26,27,27,27- $^2\text{H}_7$]Cholesterol (cholesterol- d_7) was purchased from CDN Isotopes (Pointe-Claire, QC, Canada). Soybean oil (Bakers & Chefs, Sam's Club) was purified as described elsewhere [3] to reduce the content of phytosterols. [5,6,22,23- $^2\text{H}_4$]sitostanol (sitostanol- d_4) was obtained from Medical Isotopes (Pelham, NH, USA). The phytosteryl glycosides were partially purified from lecithin granules (Vitamin Shoppe, North Bergen, NJ, USA) as previously described [19]. Crude phytosteryl glycosides were then dissolved in hexane and applied to a Supelclean LC-Si SPE column (Sigma, St Louis, MO, USA) that had been conditioned with hexane. The column was eluted with 30% ethyl acetate in

hexane, followed by 50% ethyl acetate in hexane. The material in the second elution was ASG without free phytosterols, phytosteryl esters, or phospholipids. ASG contained 48.5% phytosterols by weight. Phytosteryl esters (PSE) were derived from soy and 60.6% of the mass was the phytosteryl moiety. ASG and SG for HPLC standards were obtained from Matreya (Pleasant Gap, PA, USA).

Mice

Male C57BL/6J mice (5 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a room maintained at 24°C with a 12:12 h light–dark cycle (6:00 a.m.–6:00 p.m.). All mice were fed first on Purina mouse chow 5053 containing 4.5% fat, 20.0% protein, and 54.8% carbohydrate (LabDiet) for one week before switching to the sterol-free diet. All animal procedures were performed in accordance with guidelines of Washington University's Animal Studies Committee and approved by the Institutional Animal Care and Use Committee of Washington University (study protocol: 20070024).

Effect of a Single-Dose ASG/PSE on Cholesterol Absorption Over a Three-Day Period

Mice (5 each for control, PSE, or ASG) were fed a special cholesterol and phytosterol-free diet [22] for two weeks to minimize the effects of dietary sterols. Each mouse was gavaged with 0.5 mL purified soybean oil containing 40 µg cholesterol-d₇ and 10 µg sitostanol-d₄. The oil also contained 1.0 mg phytosterols delivered as either phytosteryl esters or glycosides or no addition (control). Stool samples from each mouse was collected individually in a cage with wire floor for three days. Mice were approximately eight weeks old at the time of experimentation.

Single-Time-Point Oral Study

Male C57BL/6J mice were equilibrated on the sterol-free diet and then fasted for 2 h before gavage of 0.5 mL purified soybean oil with 40 µg cholesterol-d₇ containing 6.1 mg phytosterols given as either phytosteryl esters (PSE, $n = 5$) or phytosteryl glycosides (ASG, $n = 5$), or oil only (control, $n = 5$). Mice were sacrificed 4 h after gavage. Blood was obtained by heart puncture and centrifuged at 8,000 rpm for 5 min to obtain serum. The liver and small intestine were removed and the small intestine was divided into four equal segments, luminal contents were washed out and collected with ice-cold PBS. After the small bowel was opened longitudinally, the mucosa was extruded from the bowel wall by exerting pressure using a microscopic slide.

Gas Chromatography–Mass Spectrometry (GC–MS)

For the cholesterol absorption study, lipids were extracted from stool samples by the Bligh and Dyer method [23], hydrolyzed in alkali, and the neutral sterols were extracted into petroleum ether, dried, and converted to penta-fluorobenzoyl esters [24]. The derivatized material was analyzed for cholesterol-d₇, coprostanol-d₇, and sitostanol-d₄ by negative-ion chemical ionization gas chromatography–mass spectrometry (GC–MS) [14]. For the single-time-point oral study, 40 µg sitostanol-d₄ was added as an internal recovery standard and lipids were extracted [23] from liver, lumen washings, and mucosa; aliquots of extracted lipids were processed by both acid and alkaline hydrolysis as described elsewhere [25] and analyzed by GC–MS [26]. Plasma was analyzed as described for liver, lumen washings, and mucosa without undergoing lipid extraction. Acid hydrolysis liberates free phytosterols from ASG and SG whereas alkaline hydrolysis does not.

ASG/SG Analysis

The ASG/SG contents of luminal and mucosal samples and stool samples were analyzed by HPLC with an evaporative light scattering detector, exactly as described elsewhere [27]. A

standard curve using ASG and SG standards was constructed and used to calculate the amount of ASG and SG in the samples.

Statistical Analysis

Data are reported as means $\pm$ SEM. All statistical analyses were performed with SAS (version 9.2; Cary, NC, USA). The Proc Mixed procedure was used to analyze treatment effects on cholesterol absorption and cholesterol-d₇ levels in liver and plasma with Tukey adjustment for multiple comparisons. Proc Mixed was also used to analyze the independent effect of treatments and quarters and their interactions. Statistical significance was accepted if $P < 0.05$, or otherwise as indicated.

Results

Purity of Phytosteryl Glycosides

Phytosteryl glycosides were obtained from lecithin granules by removing most of the phospholipids and neutral sterols [19]. To further increase purity the material was passed through a Supelclean LC-Si SPE column. Thin layer chromatographic analysis showed no detectable sterols or phospholipids (data not shown). The purified phytosteryl glycosides contained mostly ASG (92.5%) and a smaller fraction of SG (7.5%) by HPLC. Campesterol, stigmaterol, and sitosterol accounted for 27.3 ± 0.5 , 21.4 ± 0.2 , and $51.6 \pm 0.8\%$ of total phytosterols, respectively, for the phytosteryl esters; corresponding values for the phytosteryl glycosides were 21.3 ± 0.8 , 17.5 ± 0.5 , and $53.9 \pm 1.2\%$.

Effect of ASG on Percent Cholesterol Absorption in Mice

To determine whether phytosteryl glycosides were bioactive in reducing cholesterol absorption efficiency [19], we gavaged mice with ASG (1.0 mg phytosterols) solubilized in purified soybean oil. PSE (1.0 mg phytosterols) was administered in the same way as an experimental positive control and purified soybean oil alone was given to another group as a negative control. As expected, relative to control, PSE reduced cholesterol absorption by $40 \pm 6\%$ in a three-day fecal recovery experiment (Fig. 1). ASG reduced cholesterol absorption by $45 \pm 6\%$ when compared with control. The difference between ASG and PSE treatment groups was not statistically significant.

Single-Time-Point Oral Study

To explore the site where ASG reduced cholesterol absorption efficiency, groups of mice were gavaged with soybean oil containing ASG (6.1 mg phytosterols), PSE (6.1 mg phytosterols), or no addition and sacrificed 4 h later. Figures 2 and 3 report the levels of phytosterols, ASG, and SG found in the gut, lumen, and mucosa by quarter of small intestine. When no phytosterols were included in the gavage the measured phytosterol levels were very low, demonstrating the effectiveness of the prior two-week low-phytosterol baseline diet in reducing background. When either ASG or PSE was given, the total phytosterols found in the lumen (which included those present in the form of ASG and SG) were elevated and there was a significant treatment effect on total luminal phytosterols ($P = 0.0088$, Fig. 2a). There was no significant relation of intestinal quarter to luminal phytosterols ($P = 0.54$). Figure 2b shows that phytosterols accumulated in the mucosa after both ASG and PSE gavage. Total phytosterol content was largest in quarters 3 and 4 and was similar for the two phytosterol forms. There were statistically significant and independent effects of quarter ($P = 0.012$) and treatment ($P = 0.0039$) on total mucosal phytosterols.

Further studies were performed to determine the chemical form of phytosterols present in lumen and mucosa after ASG treatment. Differential hydrolysis strongly suggested that they

were present as glycosides. While combined acid + alkaline hydrolysis enables total phytosterols to be measured, alkaline hydrolysis alone does not liberate free phytosterols from glycosides to be measured by GC–MS. Phytosterols from lumen and mucosa after ASG treatment determined after alkaline-only hydrolysis were very low and similar to those in the control group (data not shown), suggesting that the total phytosterols reported in Fig. 2 after ASG treatment were derived from glycosylated phytosterols. This was confirmed by direct HPLC measurement of ASG and SG in intestinal fractions (Fig. 3; Table 1). As expected, after PSE or control gavage there was no detectable ASG or SG in the lumen or mucosa. However, after ASG gavage both ASG and SG were present in lumen and mucosa. The amount of SG exceeded that of ASG in all quarters, showing active but incomplete hydrolysis of the fatty acid of ASG to yield the deacylated product SG. In other experiments in which stools were collected for three days and analyzed by HPLC, the biological hydrolysis of ASG to SG was complete (Table 1). Recovery of SG in stool samples was $95.3 \pm 1.9\%$ of the ASG given. No detectable SG was found in stool samples from control or PSE groups.

ASG administration was associated with less systemic absorption of total phytosterols than PSE (Fig. 4). Four hours after gavage, total liver phytosterols were increased $34.0 \pm 8.5\%$ (SEM) over control with PSE but only $2.7 \pm 4.7\%$ with ASG. Plasma phytosterol levels did not change significantly with either treatment.

ASG had a large inhibitory effect on absorption of cholesterol- d_7 into plasma and liver at 4 h (Fig. 5). Compared with control, ASG reduced plasma cholesterol- d_7 by $90 \pm 3.7\%$ (SEM) and liver cholesterol- d_7 by $86.2 \pm 3.8\%$. Reductions by PSE of $51.4 \pm 19.4\%$ in plasma and $45.9 \pm 25.7\%$ in liver were more variable and not statistically significant.

Discussion

Extensive research has demonstrated that pure phytosterols, when properly formulated, reduce cholesterol absorption efficiency and plasma LDL cholesterol in animals and in humans. On the other hand, there is little published research on the physiological effects of phytosteryl glycosides. Recently we have demonstrated that ASG reduced cholesterol absorption efficiency in humans [19]. In the current study, ASG obtained from soybean-derived lecithin, was further purified and used to determine effects on cholesterol absorption and on uptake and cleavage of ASG in mouse intestine. Consistently, ASG reduced cholesterol absorption *in vivo* in mice, as observed in humans. ASG seemed to act locally in the gut lumen and mucosa with very little absorption into the systemic circulation. Furthermore, cleavage of glycosidic bonds was not required for biological activity of ASG. Most of the fatty acid moieties of ASG were removed, converting them to SG whereas the glycosidic bond remained intact.

Consistent with the known reduction in cholesterol absorption in humans, ASG reduced cholesterol absorption by 45% as determined by a three-day procedure when compared with the negative control (Fig. 1), indicating that ASG was bioactive in mice. PSE, the experimental positive control, reduced cholesterol absorption by 40% (Fig. 1). In a shorter time frame of 4 h, cholesterol d_7 , gavaged together with ASG in purified soybean oil, was subsequently found to be significantly reduced in liver and plasma compared with control (Fig. 5). These results show that ASG acts rapidly to reduce both the rate of cholesterol transport and the overall extent of cholesterol absorption.

Because standard rodent chow diets are rich in phytosterols, we used a novel cholesterol and phytosterol-free diet to enable better assessment of phytosterol absorption. Total phytosterols in the liver of mice given PSE were significantly higher than in those of control

or ASG mice (Fig. 4a). In contrast, total phytosterols in the liver or plasma of mice gavaged with ASG were similar to those of the control (Fig. 4a, b), indicating that ASG was not absorbed into the systemic circulation. Our finding agrees with results from a study in which [4-¹⁴C]sitosteryl β -D-glucoside administered intragastrically to rats was not found after 3 h in liver, kidney, heart, lungs, spleen, muscle, brain, adipose tissue, or blood [21]. The same study fed unlabeled sitosteryl β -D-glucoside at 0.5 g/kg body weight to rats for four weeks and neither sitosteryl β -D-glucoside nor sitosterol or sitosteryl esters were found in significant amounts in tissues outside the alimentary canal during the entire feeding period. It is therefore unlikely that ASG enters the systemic circulation.

ASG seemed to act locally in the lumen or in the mucosa or both. After PSE treatment total phytosterols seemed to accumulate in the lumen across the quarters (Fig. 2a), consistent with the established mechanism of action of interfering with micellar cholesterol formation in the lumen. Total phytosterols in the mucosa from the PSE group increased rapidly from the second quarter onward, indicating uptake of phytosterols into mucosa and possible action of phytosterols inside the mucosa (Fig. 2b), albeit present at a much lower level compared with luminal total phytosterols. After ASG treatment, total phytosterols in the mucosa increased from the second quarter onward (Fig. 2), similar to the pattern with PSE. Direct HPLC measurement confirmed that the accumulated phytosterols were in the form of ASG and SG (Fig. 3).

Our results strongly suggest that the bioactive components reducing cholesterol absorption are ASG or SG, but not the free phytosterols. Most of the phytosterol glycosides (92.5%) administered were in the form of ASG. Yet both ASG and SG were abundant in the lumen and mucosa in 4 h, indicating that ASG was hydrolyzed to SG in these locations. Consistent with the previous report that ASG was cleaved to SG in vitro by pancreatin and cholesterol esterase [20], most of the ASG was recovered as SG in stool samples three days after gavage, indicating that cleavage of ASG to SG was complete in three days. The complete recovery of ASG as fecal SG also indicates that SG were not hydrolyzed to free phytosterols. Furthermore, total phytosterol levels in the liver or plasma were similar to the respective levels in the control. This also is in agreement with the finding that only a small fraction of [4-¹⁴C]sitosteryl β -D-glucoside (<0.5%) was detected in the form of sitosterol and sitosteryl esters in the duodenum, jejunum, ileum, and stomach [21]. Therefore, cleavage of the glycosidic bond seems to not be required for the biological activity of ASG in mice.

In summary, the results of this study have demonstrated that ASG is bioactive in mice. ASG was taken up rapidly into the mucosa 4 h after oral gavage. ASG was cleaved to SG so that most of the ASG given was present in the form of SG in the lumen and mucosa. ASG administered was recovered quantitatively from stool samples as SG. There was little systemic absorption of ASG into the plasma or liver and ASG/SG seemed to act locally in both the lumen and mucosa without being significantly absorbed into the systemic circulation. Bioactive in reducing cholesterol absorption in mice and humans, phytosteryl glycosides from either natural or synthetic sources could potentially be used for the treatment of hypercholesterolemia. Phytosteryl glycosides must be measured and taken into account when assessing the effects of phytosterols in the diet and for treatment of hypercholesterolemia.

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Abbreviations

ASG	Acylated sterol glycosides
SG	Sterol glycosides
PSE	Phytosterol esters
GC–MS	Gas chromatography/mass spectrometry

Fig. 1.

Three-day study: effect of ASG on percent cholesterol absorption in mice. Mice were gavaged with purified soybean oil containing ASG (1.0 mg phytosterols), PSE (1.0 mg phytosterols), or no addition ($n = 5$ for each group). Percent cholesterol absorption was determined as described in "Materials and Methods". Bars represent means $\pm$ SE. Significance of differences between treatments is indicated by **($P < 0.01$)

Fig. 2.

Single-time-point study: total phytosterols in lumen (panel **a**) and mucosa (**b**). Mice ($n = 5$ for each group) were fasted for 2 h before gavage of purified soybean oil with cholesterol- d_7 containing ASG (6.1 mg phytosterols), PSE (6.1 mg phytosterols), or no addition. Mice were sacrificed 4 h later, the small intestine was cut into four equal quarters (quarter 1: proximal, quarter 4: distal), and mucosa was scraped after removal of luminal contents. Levels of total phytosterols relative to natural cholesterol in the lumen and mucosa were determined by GC-MS as described in “Materials and Methods”. Values are means $\pm$ SE. Statistical significance is indicated by [†]($P < 0.05$) or [‡]($P < 0.01$) (between control and PSE) and by ^{*}($P < 0.05$) (between control and ASG)

Fig. 3.

Single-time-point study: ASG and SG in lumen and mucosa. Mice ($n = 5$ for each group) were fasted for 2 h before gavage of purified soybean oil with cholesterol- d_7 containing ASG (6.1 mg phytosterols), PSE (6.1 mg phytosterols), or no addition. Mice were sacrificed 4 h later, the small intestine was cut into four equal quarters (quarter 1: proximal, quarter 4: distal), and mucosa was scraped after removal of luminal contents. The levels of directly measured ASG and SG relative to natural cholesterol in lumen and mucosa were determined as described in "Materials and Methods". Values are means $\pm$ SE. Significance is indicated by * ($P < 0.05$) or ** ($P < 0.01$) (between control and ASG) and by † ($P < 0.05$) or ‡ ($P < 0.01$) (between PSE and ASG). Symbols for control and PSE overlap near zero

Fig. 4. Single-time-point study: total phytosterols in liver (a) and plasma (b). Mice ($n = 5$ for each group) were fasted for 2 h before gavage of purified soybean oil with cholesterol-d₇ containing ASG (6.1 mg phytosterols), PSE (6.1 mg phytosterols), or no addition and were sacrificed 4 h later. Levels of total phytosterols in liver and plasma expressed relative to natural cholesterol were determined as described in the “Materials and Methods”. Values are means $\pm$ SE. Significance is indicated by * ($P < 0.05$) or ** ($P < 0.01$)

Fig. 5. Single-time-point study: cholesterol-d₇ in plasma (**a**) and liver (**b**). Mice ($n = 5$ for each group) were fasted for 2 h before gavage of purified soybean oil with cholesterol-d₇ containing ASG (6.1 mg phytosterols), PSE (6.1 mg phytosterols), or no addition. Mice were sacrificed 4 h later and levels of cholesterol d₇ in plasma and liver were determined as described in “Materials and Methods”. Values are means $\pm$ SEM. Significance is indicated by **($P < 0.01$)

Table 1

Phytosterol recovery in the form of sterol glycosides from stool samples three days after gavage

	Control (<i>n</i> = 5)	PSE (<i>n</i> = 5)	ASG (<i>n</i> = 5)
Total phytosterol gavaged (μg)	27.9 ± 0.6	1034.2 ± 37.3	852.1 ± 19.7
Phytosterols contained in SG (μg)	ND	ND	810.8 ± 11.7
Recovery rate in the form of SG (%)	NA	NA	95.3 ± 1.9

C57BL/6J male mice (nine weeks old) were gavaged with 0.5 mL soybean oil alone (control, *n* = 5), 0.5 mL soybean oil with phytosteryl esters (PSE, *n* = 5) containing 1.0 mg phytosterols, and 0.5 mL soybean oil with phytosteryl glycosides (ASG, *n* = 5) with 1.0 mg phytosterol equivalents. SG was determined as described in "Materials and Methods"