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SCIENCE DIRECT

Metabolism Clinical and Experimental 55 (2006) 292–299

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat

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Received 1 July 2005; accepted 5 August 2005

Abstract

Plant sterols compete with cholesterol (cholest-5-en-3β-ol) for intestinal absorption to limit absorption and lower plasma concentrations of cholesterol. Stigmasterol (24-ethyl-cholesta-5,22-dien-3β-ol; Δ^{22} derivative of sitosterol [24-ethyl-cholest-5-en-3β-ol]), but not campesterol (24-methyl-cholest-5-en-3β-ol) and sitosterol, is reported to inhibit cholesterol biosynthesis via inhibition of sterol Δ^{24}-reductase in human Caco-2 and HL-60 cell lines. We studied the effect of feeding 0.5% stigmasterol on plasma and liver sterols and intestinal cholesterol and sitosterol absorption in 12 wild-type Kyoto (WKY) and 12 Wistar rats. After 3 weeks of feeding, cholesterol and sitosterol absorption was determined in 6 rats from each group by plasma dual-isotope ratio method. After 3 more weeks, plasma and hepatic sterols and hepatic enzyme activities were determined in all rats. After feeding stigmasterol, baseline plasma cholesterol was 1.3 times and plant sterols 3 times greater in WKY compared with Wistar rats. Stigmasterol feeding lowered plasma cholesterol by approximately 11%, whereas plasma campesterol and sitosterol levels were virtually unchanged in both rat strains, and stigmasterol constituted 3.2% of plasma sterols in WKY rats and 1% in Wistar rats. After 6 weeks of feeding, cholesterol and sitosterol absorption decreased 23% and 30%, respectively, in WKY, and 22% and 16%, respectively, in the Wistar rats as compared with untreated rats. The intestinal bacteria in both rat strains metabolized stigmasterol to mainly the 5β-H stanol (>40%), with only small amounts of 5α-H derivative (approximately 1.5%), whereas the C-22 double bond was resistant to bacterial metabolism. Hepatic stigmasterol levels increased from 11 μg/g liver tissue to 104 μg/g in WKY rats and from 5 μg/g liver tissue to 21 μg/g in Wistar rats. 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity was suppressed 4-fold in the WKY and almost 1.8-fold in Wistar rats, cholesterol 7α-hydroxylase activity was suppressed 1.6-fold in the WKY and 3.5-fold in Wistar rats, whereas cholesterol 27-hydroxylase activity was unchanged after feeding. In conclusion, stigmasterol, when fed, lowers plasma cholesterol levels, inhibits intestinal cholesterol and plant sterol absorption, and suppresses hepatic cholesterol and classic bile acid synthesis in Wistar as well as WKY rats. However, plasma and hepatic incorporation of stigmasterol is low.

1. Introduction

The intestinal absorption of cholesterol (cholest-5-en-3β-ol) and dietary plant sterols is a complex process that is regulated at multiple levels. Thus, the dietary steryl ester must first be hydrolyzed to enter the enterocyte brush border membrane (solubilized in a mixed micelle) from the intestinal lumen. At the brush border, the free sterol is taken up and reesterified in the enterocyte and is transported to the plasma in chylomicrons from where it is efficiently taken up by the hepatocytes in the form of chylomicron remnants. Once in the hepatocytes, the sterols are metabolized, secreted into the bile, and in part, transported back into the plasma in the very low-density lipoproteins and high-density lipoproteins. In addition, biliary excretion of sterols regulates their intestinal levels that affect absorption. Thus, intestinal absorption of sterols can be regulated at the level of intestinal lumen, brush border, or biliary secretion.

Plant sterols (campesterol [24-methyl-cholest-5-en-3β-ol] and sitosterol [24-ethyl-cholest-5-en-3β-ol]) are structurally similar to cholesterol, differing only in the addition of a substituent at C-24 of side chain, with campesterol having a methyl group and sitosterol having an ethyl group at C-24

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doi:10.1016/j.metabol.2005.08.024

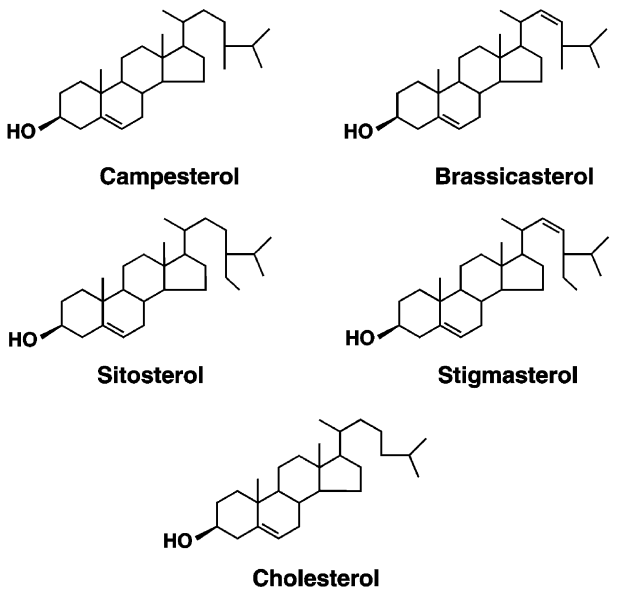

Fig. 1. Structures of cholesterol and plant sterols.

(Fig. 1). However, despite this small structural difference, plant sterols are recognized in the enterocyte and poorly transported so that their absorption is several folds lower than that of cholesterol [1-7]. Recent studies have shown that the adenosine triphosphate–binding cassette subfamily G transfer proteins, ABCG5 and ABCG8, located in the enterocyte, work together to limit intestinal absorption by promoting the secretion of cholesterol and plant sterols back to the intestinal lumen and promote their hepatic secretion [8-10]. The noncholesterol sterols are preferentially reverse-secreted out of the enterocyte into the gut lumen and also into bile as compared with cholesterol, which limits their body pool [11]. In the liver, plant sterols are poor substrates for 7α-hydroxylation, so they are secreted into the bile unchanged [12]. In the intestine, plant sterols compete with cholesterol for micellar solubilization and reduce cholesterol in the micelle and thereby interfere with cholesterol absorption [13]. More recently, it has been shown that the 5α-H–saturated derivative of sitosterol (sitostanol [24-ethylcholestan-3β-ol]) is even more poorly absorbed from the intestine than sitosterol [14], thereby suggesting that saturation of Δ^5 double bond results in a sterol that is less well absorbed and may perhaps better interfere with intestinal sterol absorption. There has been considerable interest recently in plant sterols/stanols as agents to reduce plasma cholesterol.

Recently, we fed a plant sterol mixture obtained from tall oil that contains approximately 15% stigmasterol (24-ethylcholesta-5,22-dien-3β-ol) in addition to campesterol and sitosterol to 2 species of rats, Wistar and wild-type Kyoto (WKY) [15]. It was found that plasma incorporation of stigmasterol was significantly lower than that of campesterol and sitosterol in agreement with earlier studies in the rabbit where it was shown that stigmasterol was not absorbed [16], whereas the lymphatic absorption of stigmasterol has been shown to be similar to that of sitosterol when administered intragastrically to rat [17]. Thus, we considered that stigmasterol was discriminated against more strongly for intestinal absorption and hepatic secretion than sitosterol and might competitively inhibit cholesterol and plant sterol absorption while simultaneously suppressing hepatic cholesterol synthesis. Although there are several reports on the intestinal absorption and metabolism of campesterol and sitosterol [1,18,19], little is known about the fate of stigmasterol in humans and animals [20]. In this report, we fed 0.5% stigmasterol in rat chow to 2 species of rats, Wistar and WKY, and studied its plasma and hepatic levels, biliary secretion, and intestinal metabolism and its effect on hepatic cholesterol and bile acid synthesis and on cholesterol and sitosterol absorption.

2. Experimental

2.1. Materials

Stigmasterol was purchased from Sigma-Aldrich (St. Louis, MO). Reference standards of sterols for gas-liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC-MS) were obtained from Steraloids (Newport, RI). Sil-Prep (hexamethyldisilazane/trimethylchlorosilane/pyridine, 3:1:9) used for preparation of trimethylsilyl (TMS) ether derivatives of the sterols was purchased from Alltech Associates (Deerfield, IL). All other chemicals and solvents were purchased from Aldrich Chemicals (Milwaukee, WI) and Sigma.

2.2. Animals

Male 6-week-old Wistar and WKY rats were purchased from Charles River, (Newfield, NJ). Rats were quarantined for 1 week before start of the experiment. The WKY rats are derived from Wistar rats and are shown to have elevated plant sterol levels on regular rat chow [20]. Scoggan et al [21] and Yu et al [22] have recently shown that WKY rats possess a homozygous guanidine to thymine transversion in exon 12 of the *Abcg5* gene, which is involved with limiting plant sterol absorption, and results in the substitution of a conserved glycine residue for a cysteine amino acid in the extracellular loop between the fifth and sixth membrane-spanning domains of the half-transporter, serolin-1.

2.3. Stigmasterol feeding

Male Wistar and WKY rats were divided into 2 groups of 6 rats each: group A was fed with basic rat chow, whereas group B was fed with basic rat chow fortified with 0.5% stigmasterol. Rats consumed approximately 20 to 30 g/d of basic rat chow or chow supplemented with plant sterol. After 3 weeks of feeding stigmasterol-rich diet, 6 rats from each group were given [3α-^{3}H]sitosterol intravenously and [23-^{14}C]sitosterol orally, whereas the other 6 rats from each group were given [3α-^{3}H]cholesterol intravenously and [4-^{14}C]cholesterol orally, and 100 μL blood was

collected via tail puncture on days 1, 2, 3, and 5 after labeling. The blood sample was taken in a vial containing 1 mL absolute ethanol and 3 mL of *n*-hexane, vortexed for 2 minutes, and centrifuged at 1000*g* for 5 minutes. The clear, colorless supernatant was subjected to radioactivity counting. Feeding was continued for 3 more weeks, and feces were collected for the last 3 days and freeze-dried. On the last day of feeding, 3 rats from each strain, either on basic rat chow or on the experimental diet, were anesthetized, bile fistulas were created, and bile was collected for 1 hour. All rats were then killed, blood was drawn via heart puncture, and plasma was collected and stored at −20°C for sterol analysis, whereas liver was collected and immediately stored at −70°C for sterol measurement and determination of enzyme activities.

The animal protocol was approved by the Subcommittee on Animal Studies at the Veterans Affairs Medical Center and by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey.

2.4. Animal diet

Basic rat chow (Charles River) contained 0.21 mg/g plant sterols. The experimental diet containing 0.5% stigmasterol was prepared at Charles River by mixing 0.5% stigmasterol by weight to rat chow. The stigmasterol composition in the basic and experimental chow was confirmed by GLC.

2.5. Methods

Thin-layer chromatography was carried out on silica gel G plates (Analtech, Newark, DE) in a solvent system of chloroform or benzene/chloroform (4:1), and plates were allowed to develop to a height of 20 cm. Plates were then sprayed with phosphomolybdic acid (3% in isopropanol) followed by sulfuric acid (10%) and heated for 2 minutes at 110°C to visualize the various spots due to the sterols and their metabolites. For preparative thin-layer chromatography, reference standards of sterols/stanols or their oxo derivatives were applied on the side of the plate. After the plate was developed in the appropriate solvent system, these spots were visualized by spraying with phosphomolybdic acid and sulfuric acid and heating at 110°C, making sure that rest of the plate was covered with an aluminum foil. Bands corresponding to the appropriate compounds were marked, silica gel was scraped, and compounds were eluted with chloroform/methanol (9:1; 20 mL). After complete removal of solvents, the residue was used for GLC or GC-MS for identification of the compound.

2.6. Trimethylsilylether derivatization of sterols and stanols

Aliquots of the biological extracts that contained the sterols (and stanols) were treated with 100 μL of Sil-Prep for 30 minutes at 55°C. Solvents were evaporated at 55°C under nitrogen, the reaction product was dissolved in 100 μL hexane, and an aliquot was used for GLC [23].

2.7. Gas-liquid chromatography

A Hewlett-Packard model 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused silica CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m × 0.22 mm ID) (Chrompack, Raritan, NJ), and helium was used as the carrier gas [23]. The GC operating conditions were as follows: injector and detector temperatures were 260°C and 290°C, respectively. After injection, oven temperature was kept at 100°C for 2 minutes and then programmed at a rate of 35°C/min to a final temperature of 268°C.

2.8. Gas chromatography–mass spectrometry

Mass spectra of the various sterols, bile acids, and fatty acids, whenever needed, were carried out on a Hewlett-Packard Model 5972A mass-selective detector coupled to a model 6890 gas chromatograph using a 25-m CP-Sil-5 CB capillary column.

2.9. Sterol determination

Plasma (100 μL), bile (50 μL), or liver tissue (50-100 mg, exactly weighed) plus internal standard (coprostanol [5β-cholestan-3β-ol], 20 μg in 200 μL ethanol) were taken in 10 mL of 1 N ethanolic sodium hydroxide and heated at 70°C for 1 hour. After cooling, the neutral sterols and stanols were extracted with *n*-hexane (3 × 30 mL). Hexane was

Table 1
Plasma sterols in Wistar and WKY rats before and after feeding 0.5% stigmasterol for 6 weeks

Treatment	Cholesterol	Campesterol	Stigmasterol (mg/dL)	Sitosterol	% Plant sterols	Stigmasterol as % plant sterols
Wistar rats						
Untreated	44.7 ± 5.1	1.1 ± 0.3	0.01 ± 0.01	1.0 ± 0.3	4.5 ± 0.9	0.5
Stigmasterol-fed	39.9 ± 4.2*	1.0 ± 0.3*	0.4 ± 0.3**	0.8 ± 0.2*	5.2 ± 1.5	18.2
WKY rats						
Untreated	58.6 ± 3.3***	2.3 ± 0.2***	0.13 ± 0.14***	3.6 ± 0.6***	9.3 ± 1.2	2.2
Stigmasterol-fed	52.2 ± 3.3**	2.1 ± 0.2*	1.9 ± 0.3**	3.2 ± 0.4*	12.1 ± 1	26.4

Six rats of each strain were used for each feeding regimen. Plasma (100 μL) was saponified with 1 N ethanolic sodium hydroxide, sterols were extracted with *n*-hexane, and an aliquot was used as the TMS derivatives for quantitation by GLC as described in the Experimental section.

* P = NS when compared with untreated rats of same strain.

** $P < .05$ when compared with untreated rats of the same strain.

*** $P < .05$ when compared with untreated Wistar rats.

Table 2
Biliary sterols in Wistar and WKY rats before and after feeding experimental diets for 1 month

Treatment	Cholesterol	Cholesterol precursors[a]	Campesterol (μg/mL bile)	Stigmasterol	Sitosterol	% Plant sterols	Stigmasterol as % plant sterols
Wistar rats							
Untreated	63.7 ± 6.3	0.5 ± 0.3	2.9 ± 0.7	–	2.8 ± 0.8	8.2	ND
Stigmasterol-fed	57.4 ± 7.4*	0.7 ± 0.3*	2.8 ± 0.7*	1.3 ± 0.3	3.1 ± 0.6*	11.0	18.1
WKY rats							
Untreated	56.2 ± 5.6	0.60 ± 0.4	1.9 ± 0.6	0.4 ± 0.2	2.0 ± 0.6	7.0	9.3
Stigmasterol fed	52.8 ± 4.2*	0.8 ± 0.3*	1.7 ± 0.6*	1.2 ± 0.3**	2.0 ± 0.5*	9.4	24.5

Values are reported as mean ± SD. ND indicates not determined. Bile fistula was created in 3 male rats from each strain, on basic rat chow, or on the last day of feeding of the experimental diet, and bile was collected for 1 hour (0.8 mL bile was obtained from Wistar rats and 0.45 mL from WKY rats). Bile (100 μL) was heated for 1 hour at 70 °C with 2 mL 1 N ethanolic NaOH. Sterols were extracted with *n*-hexane and converted into TMS derivatives, and a one-hundredth aliquot was injected onto the GLC column. Gas-liquid chromatography operating conditions used were as described in the Experimental section. Each analysis was carried in duplicate, and values reported are average for each treatment.

[a] Desmosterol + lathosterol.

* P = NS when compared with untreated rats of same strain.

** $P < .05$ when compared with untreated rats of same strain.

evaporated under vacuum at 40°C, and the residual product was transferred to a small vial and subjected to TMS ether formation. After evaporation of excess reagent at 50°C under a current of nitrogen, the residue was dissolved in 100 μL hexane, and 1 μL was injected onto the GLC column.

For analysis of the stool, the 3-day freeze-dried feces were ground to a fine powder, an aliquot (10-15 mg, exactly weighed) was taken in a small screw-cap vial, and the fecal sterols were converted to their TMS ether derivatives by heating with Sil-Prep (200 μL) at 55°C for 30 minutes. The pyridine in Sil-Prep acted as extraction solvent for the fecal sterols. After centrifugation at 1000*g* for 10 minutes, 2 μL of the clear supernatant was subjected to GLC [24]. Structures of the fecal sterols and their metabolites were determined by GC-MS as described previously [15].

2.10. Assay for 3-hydroxy-3-methylglutaryl coenzyme A reductase activity

Hepatic microsomal and mitochondrial fractions were prepared by differential ultracentrifugation [25], and the protein concentrations were determined by the Bradford [26] method. Microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was measured according to the method described by Nguyen et al [27].

2.11. Assay for cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase activities

The activities of microsomal cholesterol 7α-hydroxylase (cyp7A1) and mitochondrial cholesterol 27-hydroxylase (cyp27A1) were measured by the stable-isotope dilution mass spectrometry methods described previously, where [$^2H_7$]7α-hydroxycholesterol and [$^2H_7$]27-hydroxycholesterol were used as internal recovery standards, and the amounts of microsomal 7-hydroxycholesterol and [$^2H_7$]7-hydroxycholesterol and mitochondrial 27-hydroxycholesterol and [$^2H_7$]27-hydroxycholesterol were measured by GC-MS with selected ion monitoring [28].

2.12. Intestinal sitosterol and cholesterol absorption

The dual-isotope ratio method originally described by Zilversmit and Hughes [29] was used. [3α-^{3}H]Sitosterol or [3α-^{3}H]cholesterol (1 μCi, in 0.2 mL of 20% liposyn) was

Table 3
Sterols in the livers of Wistar and WKY rats before and after feeding 0.5% stigmasterol for 6 weeks

Treatment	Cholesterol	Campesterol	Stigmasterol (μg/g)	Sitosterol	% Plant sterols	Stigmasterol as % plant sterols
Wistar rats						
Untreated	2153 ± 44	35 ± 7	5 ± 1	32 ± 4	3.2 ± 0.5	6.9 ± 2.0
Stigmasterol-fed	2720 ± 113*	47 ± 6	21 ± 11*	35 ± 8	3.6 ± 1.3	19.2 ± 4.0
WKY rats						
Untreated	1911 ± 118**	76 ± 11**	11 ± 3**	97 ± 2**	8.8 ± 0.4	6.0 ± 1.7
Stigmasterol-fed	2372 ± 110*	103 ± 17*	104 ± 10*	102 ± 8	11.5 ± 0.5	33.7 ± 0.9

Values are reported as mean ± SD. Six rats of each strain were used for each feeding regimen. Approximately 100 mg of liver tissue was exactly weighed and heated for 1 hour at 70°C with 2 mL of 1 N ethanolic NaOH. Sterols were extracted with *n*-hexane and converted into TMS derivatives, and a one-hundredth aliquot was injected onto the GLC column. Gas-liquid chromatography operating conditions used were as described in the Experimental section.

* P <.05 when compared with untreated rats of same strain.

** $P < 0.05$ when compared with untreated Wistar rats.

Table 4
Major sterols and their metabolites in rat feces before and after feeding 0.5% stigmasterol

Steroid	Wistar rats		WKY rats	
	Untreated	Plant sterol fed	Untreated	Plant sterol fed
Coprostanol	2.15 ± 1.00	2.80 ± 3.20	1.46 ± 1.22	2.60 ± 1.55
Epi-coprostanol	0.50 ± 0.30	0.80 ± 0.80	0.40 ± 0.30	0.40 ± 0.30
Cholesterol	4.50 ± 1.50	5.42 ± 2.30	2.10 ± 1.10	3.00 ± 1.00
Cholestanol	0.10 ± 0.10	0.22 ± 0.20	0.09 ± 0.05	0.20 ± 0.20
24-Methylcoprostanol	0.61 ± 0.30	1.00 ± 0.35	0.64 ± 0.44	0.71 ± 0.41
Campesterol	0.80 ± 0.30	1.20 ± 0.80	0.56 ± 0.22	1.05 ± 0.40
Campestanol	0.14 ± 0.12	0.15 ± 0.15	0.18 ± 0.10	0.23 ± 0.22
Δ^{22}-5β-Stigmastanol	0.51 ± 0.35	32.00 ± 6.05	0.50 ± 0.50	22.00 ± 7.50
Stigmasterol	0.37 ± 0.15	42.24 ± 12.50	0.10 ± 0.08	30.90 ± 12.85
Δ^{22}-5α-stigmastanol	ND	1.08 ± 0.35	ND	0.90 ± 0.35
24-Ethylcoprostanol	1.40 ± 0.40	1.86 ± 0.55	1.34 ± 0.25	1.25 ± 0.35
Sitosterol	3.62 ±1.20	3.93 ± 1.50	1.40 ± 0.25	1.20 ± 0.45
Sitostanol	0.49 ± 0.25	0.60 ± 0.45	0.30 ± 0.20	0.30 ± 0.20

Values are given as mg/d dry feces and are reported as mean ± SD. Feces were collected during the last 3 days from either untreated rats or form rats that were fed with the plant sterol-enriched diet. Freeze-dried homogenized feces (10-15 mg exactly weighed) was taken in a screw-cap vial and heated with Sil-Prep (200 μL) at 55°C for 30 minutes. After centrifugation at 1000g for 10 minutes, 2 μL of the clear supernatant was subjected to GLC.

injected intravenously, and [23-^{14}C]sitosterol or [4-^{14}C]cholesterol (2 μCi, in 0.5 mL of 20% liposyn) was given by gavage in the morning to each rat, and 100 μL blood was obtained on days 1, 2, 3, and 5 as described above in the Experimental section. The blood sample was taken in a vial containing 1 mL absolute ethanol and 3 mL n-hexane, vortexed for 2 minutes, and centrifuged at 1000g for 5 minutes. The clear, colorless supernatant was subjected to radioactivity counting. Percentage of absorption was calculated by determining the mean ^{14}C/^{3}H ratio in the blood and dividing by the ideal ratio:

$$\%\ absorption = \frac{[^{14}C/^{3}H\ ]\ \text{blood} \times 100}{[^{14}C/^{3}H\ ]\ \text{ideal}}$$

The ideal ratio is the total oral dose÷total injected dose, and this ratio would be obtained in the blood if 100% of the oral [^{14}C] radioactivity would be absorbed. Mean absorption values for days 1, 2, 3, and 5 were obtained, and values reported are a mean for 6 rats in each group.

2.13. Statistics

Data are reported as the mean ± SD. The statistical significance of difference between the results in different groups was evaluated by the Student t test (unpaired), and significance was accepted at the P level of less than .05.

3. Results

The aim of this study was to determine if feeding of stigmasterol, the Δ^{22} derivative of sitosterol, would interfere with cholesterol and plant sterol absorption and lower plasma sterol levels in rats and to determine the fate of the fed stigmasterol. Stigmasterol was fed in a rat chow at a concentration of 0.5% to WKY rats that are known to hyperabsorb plant sterols and to Wistar rats that were used as controls. The diet was well tolerated by the rats, and they gained weight normally. Table 1 shows plasma sterol levels in the WKY and Wistar rats. The WKY rats had higher plasma cholesterol as well as plant sterol levels as compared with Wistar rats at baseline. Stigmasterol feeding for 6 weeks did not change plasma plant sterol levels in either species, whereas the cholesterol levels decreased 11%, although a statistical significance was reached only in the WKY rats (Table 1). Stigmasterol was barely detected in untreated Wistar rats and increased to 0.4 mg/dL after stigmasterol feeding. WKY rats, which absorb larger proportions of plant sterols because of mutation in intestinal *abcg5/8*, had 0.13 mg/dL stigmasterol in their plasma at baseline, which rose to 1.9 mg/dL (3.2% of total plasma sterols and 26.4% of plasma plant sterols) after feeding. In an earlier study, Ikeda et al showed that feeding 0.5% plant sterol mixture (major stigmasterol) in rat chow increased plasma levels of plant sterol in stroke-prone hypertensive rats from approximately 10% to as much as 30% of total plasma sterols [30]. We had a similar finding when we fed a larger dose (1%) of plant sterol mixture to WKY rats, suggesting limited solubility and hence availability of plant sterols for intestinal absorption [15]. Thus, stigmasterol was less well incorporated into plasma than sitosterol. This observation is in line with our earlier feeding study where we fed a 1% plant sterol mixture that contained approximately 15% stigmasterol (approximately 50 mg/d stigmasterol) and found that stigmasterol constituted 0.5% of total plasma sterols and 1.6% of total plasma plant sterols.

Biliary sterols in both rat species are reported in Table 2. The pretreatment plant sterol levels in the Wistar and WKY rats were similar (8.2 μg/mL bile from Wistar rats vs 7.0 μg/mL bile from WKY rats) and were not much changed after stigmasterol feeding. Biliary secretion of stigmasterol was similar in both rat species, although plasma stigmasterol levels were higher in WKY rats. This suggested that like plant sterol secretion [15,30], the biliary secretion

of stigmasterol is lower in WKY rats as compared with the Wistar rats.

Table 3 shows hepatic levels of sterols in these rats. Increased cholesterol levels were seen in the livers from Wistar rats as compared with WKY rats. However, contrary to sitosterol feeding where hepatic cholesterol levels were diminished after feeding [15], stigmasterol feeding resulted in increased hepatic cholesterol levels in both rat species. As expected, hepatic plant sterol levels were increased in WKY rats and were further increased after stigmasterol feeding, the increase being due primarily to addition of stigmasterol in the hepatocytes. Stigmasterol levels were also increased in the Wistar rats from pretreatment levels, albeit to a lesser extent.

Fecal sterols were analyzed by GC-MS as described in detail in our earlier publication [15], and the major sterols and their metabolites that were characterized are reported in Table 4. The 5α- and 5β-H derivatives of cholesterol, campesterol, stigmasterol, and sitosterol were all identified [31]. Coprostanol, 24-methylcoprostanol (24-methyl-5β-cholestan-3β-ol), Δ^{22}-5β-stigmastanol (24-ethyl-5β-cholest-22-en-3β-ol), and 24-ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol) were the major metabolites of the Δ^5-sterols, cholesterol, campesterol, stigmasterol, and sitosterol, respectively. The Δ^{22} double bond in stigmasterol was left unaffected by the colonic bacteria as the excretion of 24-ethylcoprostanol, sitostanol, and sitosterol (all possible metabolites of stigmasterol with saturated side chain) did not increase appreciably after stigmasterol feeding.

The activity of hepatic HMG-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, was found to be of the same order in the Wistar and the WKY rats, but was reduced by 44% and 77%, respectively, in the Wistar and the WKY rats after feeding stigmasterol enriched diet (Table 5). Consistent with this was the finding that hepatocyte cholesterol levels were increased after stigmasterol feeding in both rat strains. The activity of cyp7A1, reflecting classic bile acid synthesis, was 35% lower in the Wistar rats as compared with the WKY rats at baseline and was reduced by 71% in the Wistar rats and 39% in the WKY rats after stigmasterol feeding. This finding is also contrary to what we found after feeding 1% plant sterol mixture (mainly sitosterol, campesterol, and stigmasterol), when cyp7A1 activity was rather increased 20% and 29%, respectively, in WKY and Wistar rats [15]. cyp27A1, which reflects alternative (acidic) bile acid synthesis, was slightly decreased in Wistar rats at baseline (P = NS), but remained unchanged after stigmasterol feeding to both rat strains (Table 5).

Table 5
Hepatic enzyme activities in Wistar and WKY rats before and after feeding 1% plant sterol-enriched diet

Rat	HMG-CoA	cyp7A1	cyp27A1
Wistar rats			
Untreated	17.9 ± 9.5	13.9 ± 0.7	10.1 ± 1.3
Stigmasterol-fed	10.1 ± 4.1*	4.0 ± 1.8**	11.2 ± 2.2*
WKY rats			
Untreated	21.8 ± 0.7	18.7 ± 1.3	13.0 ± 1.0
Stigmasterol-fed	5.4 ± 3.0**	11.5 ± 2.2**	11.1 ± 2.2*

Values are given as pmol/mg protein per minute and are reported as mean ± SD. Livers were collected immediately after rats were killed and stored at −70°C and used for measurement of enzyme activities. Each analysis was carried out in duplicate, and the average was used.

* P = NS when compared with untreated rats from same strain.

** P < .05 when compared with untreated rats from same strain.

Table 6
Cholesterol and sitosterol absorption in Wistar and WKY rats treated with 0.5% stigmasterol for 3 weeks

Rat	Absorption (%)			
	Cholesterol	% Reduction	Sitosterol	% Reduction
Wistar rats				
Untreated	73.4 ± 3.4	22.2	5.0 ± 0.3	16.0
Stigmasterol fed	57.1 ± 4.1*		4.2 ± 0.2*	
WKY rats				
Untreated	72.7 ± 3.7	22.7	8.4 ± 0.5	29.8
Stigmasterol fed	56.2 ± 3.4*		5.9 ± 0.4*	

Values are reported as mean ± SD. Six rats from each group were fed with basic rat chow or experimental diet for 3 weeks and were given [3α-^{3}H]sitosterol intravenously and [23-^{14}C]sitosterol by mouth, whereas the other 6 rats from each group were given [3α-^{3}H]cholesterol intravenously and [4-^{14}C]cholesterol by mouth. Blood (100 μL) was collected via tail puncture on days 1, 2, 3, and 5 after labeling. The blood was taken in a vial containing 1 mL absolute ethanol and 3 mL of n-hexane, vortexed for 2 minutes, and centrifuged at 1000g for 5 minutes. The clear, colorless supernatant was subjected to radioactivity counting.

* P < .05 when compared with untreated rats of the same species.

Intestinal absorption of cholesterol and sitosterol was determined in untreated and stigmasterol-fed WKY and Wistar rats by the plasma dual-isotope ratio method, and results are shown in Table 6. It was found that, in untreated rats, where plant sterol absorption would be at maximum, the absorption of ^{14}C-labeled sitosterol was similar in both rat strains (5.0% in Wistar rats vs 8.4% in WKY rats) and was suppressed 16% and 30%, respectively, in the 2 strains after 3 weeks of stigmasterol feeding. Thus, stigmasterol feeding suppressed sitosterol absorption in a manner similar to what was observed when sitosterol was fed to these rats. In addition, cholesterol absorption was found to be of the same order in the 2 rat strains (Table 6) and was reduced by approximately 22% to 23% in both strains.

4. Discussion

There is ample evidence that structural modifications in cholesterol molecule suppress its intestinal absorption. Thus, plant sterols with 1 or 2 extra carbons in the side chain and the corresponding 5α-stanols incorporate in intestinal micelle to displace cholesterol and are poorly absorbed and interfere with cholesterol absorption from the intestine. Furthermore, there is circumstantial evidence that stigmasterol, the Δ^{22} derivative of sitosterol, is more poorly absorbed than sitosterol [16,17,20]. In a recent study, we

found that feeding a plant sterol mixture to WKY rats resulted in increased activity of HMG-CoA reductase [15]. Thus, the beneficial effect of suppression of cholesterol absorption by plant sterol feeding is at least partially overcome by increased hepatic synthesis. We also found that plasma incorporation of stigmasterol when fed in the plant sterol mixture was relatively low as compared with that of campesterol and sitosterol, and its biliary secretion was relatively increased. It has been reported that stigmasterol is poorly taken up by the brush border membrane and inhibits cholesterol synthesis via competitive inhibition of sterol Δ^{24}-reductase in human Caco-2 and HL-60 cell lines, whereas both campesterol and sitosterol, with saturated side chain, were ineffective [32]. In a more recent study, Yang et al [33] found that addition of stigmasterol, but not sitosterol, inhibited SREBP-2 processing and reduced cholesterol synthesis in cultured adrenal cells from *abcg5/8* knockout mice. Stigmasterol was also shown to activate the liver X receptor in a cell-based reporter assay.

In the current study, we fed WKY and Wistar rats with a diet containing 0.5% stigmasterol and looked at stigmasterol distribution in plasma, bile, feces, and liver, and its effect on cholesterol and plant sterol absorption. As is obvious from Table 1, plasma incorporation of stigmasterol was low as compared with what was found for sitosterol in the same rat strains [30]. This suggests that only a small amount of the fed stigmasterol is available for absorption or intestinal absorption of stigmasterol is very low. Plasma levels of stigmasterol were higher in the WKY rats than Wistar controls. However, stigmasterol did not seem to change plasma campesterol and sitosterol levels in either rat species and resulted in approximately 11% reduction in plasma cholesterol levels.

The activities of HMG-CoA reductase and cyp7A1 were somewhat lower in the Wistar rats as compared with the WKY rats, and after feeding stigmasterol-enriched diet, both enzyme activities were further reduced in these rat strains. Apparently, increased hepatocyte levels of cholesterol resulting from stigmasterol feeding cause feedback inhibition and suppress HMG-CoA reductase. This reduced cholesterol synthesis further results in suppression of cholesterol 7α-hydroxylation, and the newly synthesized cholesterol being predominantly esterified and stored in the hepatocytes may not be available for 7α-hydroxylation. This is in contrast to the finding with sitosterol feeding where hepatic cholesterol is reduced after feeding and an increase in activities of these enzymes is observed. In an earlier study, Fernandez et al [32] have shown that the Δ^{22} sterols inhibit cholesterol synthesis via competitive inhibition of sterol Δ^{24}-reductase in human Caco-2 and HL-60 cell lines. We now find that stigmasterol also suppresses cholesterol synthesis via competitive inhibition of HMG-CoA reductase activity.

In a recent study, Owsley and Chiang [34] have shown that guggulsterone, a plant sterol that lowers serum cholesterol levels, strongly inhibits the human *CYP7A1* gene by activation of pregnane X receptor (PXR). The authors concluded that guggulsterone inhibits bile acid secretion from hepatocyte into bile and activates PXR to inhibit hepatic bile acid synthesis and that the reduced metabolism of cholesterol and bile acid secretion lead to increased hepatic cholesterol levels and its reduced intestinal absorption, thereby resulting in lower serum cholesterol levels. We believe that stigmasterol works similarly, and the suppression of cholesterol 7α-hydroxylation with stigmasterol feeding may be due to inhibition of *cyp7A1* gene in these rats via activation of PXR, and the reduced conversion to bile acids results in increased hepatic cholesterol levels.

The small intestine can differentiate between luminal cholesterol and plant sterols so that plant sterols are absorbed at a rate of 1/5 to 1/10 of that of cholesterol [1]. It has been suggested that the absorptive discrimination against the plant sterols is the result of greater reverse sterol transport by ABCG5/8 located in the enterocytes. The presence of the double bond in the side chain of stigmasterol makes it more difficult to pass through the enterocyte. Therefore, stigmasterol is likely to be poorly absorbed (as reflected by low plasma levels after feeding). Even still, as shown in Table 6, stigmasterol feeding results in inhibition of absorption of cholesterol and other sterols. Feeding larger amounts of stigmasterol may not increase its overall absorption and plasma and tissue levels because of its limited solubility. The side-chain double bond in stigmasterol was found to be resistant to bacterial saturation or degradation, and the fecal outputs of cholesterol, campesterol, and sitosterol and their 5β-H derivatives seemed to increase from their pretreatment levels in all animals after they were fed with stigmasterol-rich diet, suggesting interference with intestinal absorption by the fed stigmasterol.

Thus, we have shown that feeding of 0.5% stigmasterol for 6 weeks to Wistar and WKY rats significantly suppresses HMG-CoA reductase activity and results in approximately 11% reduction in plasma cholesterol levels. However, because of apparent reduced solubility, its plasma incorporation is very low and its competitive inhibition of cholesterol and plant sterol absorption is not high, so that the effect on plasma plant sterol levels is not apparent after 6 weeks of feeding. The solubility and hence absorbability of stigmasterol may increase after feeding with the fatty acid ester [35,36] and is likely to exert greater competitive inhibition of cholesterol and plant sterol absorption and thereby lower their plasma levels. An added advantage of feeding stigmasterol over sitosterol (probably as its fatty acid ester) is that stigmasterol also suppresses hepatic cholesterol synthesis, whereas sitosterol feeding results in an increase in endogenous synthesis of cholesterol so that plasma cholesterol-lowering effect of sitosterol may not reach high levels.

Acknowledgment

This work was supported by grants from the Veterans Affairs Research Service, Washington, DC, the American

Heart Association, the New Jersey Affiliate, and the National Institutes of Health grant DK 568301A.

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