Missense Mutation in Abcg5 in SHRSP Rats Does Not Accelerate Intestinal Absorption of Plant Sterols: Comparison with Wistar Rats

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Stroke-prone spontaneously hypertensive rats (SHRSP) deposit plant sterols in their bodies and have a mutation in ATP binding cassette transporter G5 (Abcg5). Lymphatic recovery rates of campesterol and sitosterol in SHRSP rats were comparable to those in Wistar rats, a strain that does not deposit plant sterols in the body and has no mutation in Abcg5. Higher absorption of stigmasterol and sitostanol was observed in SHRSP rats than in Wistar rats, but the differences between SHRSP and Wistar rats were quite small, because the absorbed amounts of these two sterols were much lower than those of campesterol and sitosterol. The in situ uptake of 3H-sitosterol and 14C-cholesterol solubilized in the bile salt micelle into intestinal mucosa was comparable between SHRSP and Wistar rats. These observations suggest that a mutation in Abcg5 does not greatly influence intestinal absorption of plant sterols in SHRSP rats, at least in comparison with Wistar rats.

Key words: ATP-binding cassette transporter G5 (ABCG5); ATP-binding cassette transporter G8 (ABCG8); intestinal absorption; plant sterols; stroke-prone spontaneously hypertensive rats (SHRSP)

Plant sterols (Fig. 1) are less absorbable than cholesterol and their deposition in the body is extremely low. However, it has been found that plant sterols are deposited in the bodies of sitosterolemia patients.¹ Several mutations in ATP binding cassette transporter G5 (ABCG5) and in ATP binding cassette transporter G8 (ABCG8) were found in sitosterolemia.¹⁻³ ABCG5 and ABCG8 mainly express in the liver and the intestine.⁴⁻⁵ In ABCG5/ABCG8-deficient mice, intestinal absorption of plant sterols was higher and biliary secretion of cholesterol was lower than in wild-type mice.⁵ Hence, it has been suggested that these transporters function as heterodimer and excrete sterols from intestinal epithelial cells to the intestinal lumen and from hepatocytes to bile.⁴

We have observed that Wistar Kyoto (WKY) rats, spontaneously hypertensive rats (SHR), and stroke-prone spontaneously hypertensive rats (SHRSP) deposited plant sterols in the body.⁶ Lymphatic absorption of a trace amount of sitosterol administered to the stomach was higher in SHRSP rats than in Wistar-King A (WKA) rats, a normal strain in which deposition of plant sterols was lower than in SHRSP rats. We concluded that one of the causes of deposition of plant sterols in SHRSP rats is acceleration of plant sterol absorption in the intestine. Since Scoggan et al.¹³ and Yu et al.¹⁴ found that WKY, SHR, and SHRSP rats have the same mutation in Abcg5, we concluded that accelerated absorption of plant sterols is caused by a malfunction of ABCG5, but no quantitative estimation of reduced excretion of plant sterols by intestinal ABCG5 and ABCG8 to the intestinal lumen in SHRSP rats has been done. In the present study, we compared the lymphatic absorption of various plant sterols between SHRSP and Wistar rats. In our previous study, WKA rats were used as a control strain.⁵ Since WKA rats are not available at present, we used Wistar rats, which do not have a mutation in Abcg5 and do not deposit plant sterols in the body.⁵

Igel et al. have proposed that plant sterols are incorporated into intestinal cells and then are secreted to the intestinal lumen via ABCG5 and ABCG8.⁹ They suggested that secretion of plant sterols into the intestinal lumen by ABCG5 and ABCG8 is an important determinant of intestinal absorption of sterols. In the present study, we also evaluated to determine whether incorporation of sitosterol and cholesterol into intestinal cells is accelerated in SHRSP rats as compared with Wistar rats.

Materials and Methods

Materials. Campesterol (purity, 94.4%), stigmasterol (97.7%), and sitosterol (98.6%) were kindly provided by Tama Biochemical (Tokyo). Sitostanol (95.9%), cholesterol (<99%), triolein, and L-α-phosphatidylcholine were purchased from Sigma (Tokyo). Sodium

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Abbreviations: ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; NPC1L1, Niemann-pick C1-like 1 protein; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone spontaneously hypertensive rats; WKA, Wistar King-A; WKY, Wistar Kyoto
taurocholate (>97%) was purchased from Nacalai Tesque (Kyoto, Japan). [22, 23(n)^3H] Sitosterol (814 GBq/mmol, Amersham, Buckingamshire, UK) was kindly provided by Kao (Tokyo). [4,14C] Cholesterol (2.0 GBq/mmol) was purchased from Amersham Pharma Biotech (Tokyo).

Cannulation of thoracic lymphatic duct. The left thoracic lymphatic duct cephalad to the cisterna chilii was cannulated as described previously. A second indwelling catheter was placed in the stomach duct. In guinea pigs, the thoracic duct was perforated to allow the lymphatic fluid to pass through the oral cavity, and a second indwelling catheter was placed in the stomach as described by Kato (Tokyo). [4-Yoshitomi, Fukuoka, Japan] were fed an AIN-93G purified diet. Since rat chow diets contain considerable amounts of plant sterols, the SHRSP rats obtained from the breeder had already deposited plant sterols in the bodies. To reduce plant sterols in the body as low as possible, lard was used as the sole dietary fat, because the plant sterol content in the lard is extremely low (sterol content in weight percent: cholesterol, 0.0062%; campesterol, 0.00078%, sitosterol, 0.0013%). These rats were subjected to surgery. The average body weights at surgery were 170–190 g for the SHRSP and Wistar rats respectively. Lipid emulsions composed of 67 mg of sodium taurocholate, 17 mg of bovine serum albumin, 67 mg of triolein, and 6.7 mg of a purified plant sterol in 1 ml were prepared by sonication. In this study, four purified plant sterols, campesterol, stigmasterol, sitosterol, and stigmasterol, were used in preparing test emulsions, and they were subjected to sterol analysis. Because slight amounts of plant sterols were detected in the lymph before the infusion of lipid emulsion, the amounts of plant sterols in the lymph before administration were used as the base line.

Total lipids in the lymph were extracted and purified by the method of Folch et al. After saponification of total lipids, the unsaponifiable matter collected was derivatized to trimethylsilyl ethers and quantified by gas-liquid chromatography using a SPELCO SPB-1 column (0.25 mm x 60m, 0.25 mm film thickness, Sigma-Aldrich Japan, Tokyo) and 5α-cholestanol (Sigma) as an internal standard.

Uptake of cholesterol and sitosterol in jejunal loops in situ (experiment 2). Eight-week-old male SHRSP rats (body weight, 170–190 g) and Wistar rats (Wistar/Kud, outbred, SPF, body weight 230–260 g, Kyudo, Fukuoka, Japan) were fasted overnight. The rats were anesthetized with pentobarbital (50 mg/kg of body weight) and kept in a chamber at 37 °C throughout the experiment. The abdomen was opened and two jejunal segments approximately 7 cm in length were ligated without disturbing the intact blood supply. The first segment was just distal to the ligament of Treitz. The second segment was 1 cm distal to the first one. Before tight closing of the segments, 0.5 ml of a micellar solution composed of 0.6 mM phosphatidylcholine, 0.05 mM cholesterol, 0.05 mM sitosterol, 18.5 kBq 14C-cholesterol, 185 kBq 3H-sitosterol, and 6.6 mM sodium taurocholate in 15 mM sodium phosphate buffer (pH 7.4) prepared by sonication was infused into the first segment through one end. The abdomen was closed and the rats were kept at body temperature for 5, 10, 20, 60, 120, and 240 min. Just before termination of incubation, the abdomen was opened again and 0.5 ml of the same micellar solution was infused into the second segment. Immediately after infusion, two segments were resected and the jejunal contents were washed out with physiological saline. The wet weights of the washed segments were recorded, and then the segments were solubilized with KOH solution (60% by wt) at 70 °C. These solubilized samples were subjected to saponification, and the radioactivity in the unsaponifiable matter was measured with a liquid scintillation counter. The radioactivity in the second segment was used as a zero-time blank.

Statistical analysis. All data were expressed as means ± SE. Student’s t-test was used, and P values of less than 0.05 were considered significant.
Table 1. Lymph Flow Rates and Lymphatic Absorption of Plant Sterols in SHRSP and Wistar Rats

<table>
<thead>
<tr>
<th>Sterols</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>Sitosterol</th>
<th>Sitostanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph flow (ml/24 h)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Wistar</td>
<td>165 ± 13</td>
<td>147 ± 13</td>
<td>174 ± 18</td>
<td>150 ± 20</td>
</tr>
<tr>
<td>SHRSP</td>
<td>76.5 ± 6.2a</td>
<td>78.3 ± 5.3a</td>
<td>64.9 ± 4.8a</td>
<td>62.5 ± 3.7a</td>
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<tr>
<td>Lymphatic absorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery for 24 h (%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Wistar</td>
<td>13.9 ± 1.6</td>
<td>0.383 ± 0.065</td>
<td>2.79 ± 0.27</td>
<td>0.242 ± 0.014</td>
</tr>
<tr>
<td>SHRSP</td>
<td>13.8 ± 2.2</td>
<td>0.554 ± 0.095</td>
<td>2.84 ± 0.41</td>
<td>0.423 ± 0.032a</td>
</tr>
<tr>
<td>Absorbed mass (μg/100 g of body weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar</td>
<td>908 ± 114</td>
<td>23.3 ± 4.0</td>
<td>183 ± 17</td>
<td>15.1 ± 0.8</td>
</tr>
<tr>
<td>SHRSP</td>
<td>911 ± 153</td>
<td>33.7 ± 5.8</td>
<td>187 ± 28</td>
<td>26.1 ± 2.1a</td>
</tr>
</tbody>
</table>

The animals were fed a diet containing 10% lard ad libitum for 4 weeks, and then were subjected to absorption study.
Data are means ± SE for eight rats per group.
*aSignificant difference between SHRSP and Wistar rats at P < 0.05 by Student’s t-test.

Results

Comparison of lymphatic recovery of highly purified plant sterols between SHRSP and Wistar rats (experiment 1)

Lymphatic 24-h recovery for campesterol and sitosterol was comparable between SHRSP and Wistar rats (Table 1). Lymphatic recovery for stigmasterol and sitostanol in SHRSP was higher than that in Wistar rats ($P = 0.08$ for stigmasterol, $P < 0.01$ for sitostanol). Compared with campesterol, the amounts of stigmasterol and sitostanol absorbed were much lower: the values were about 1/30 (Table 1).

Uptake of cholesterol and sitosterol in jejunal loops in situ (experiment 2)

Uptake of micellar cholesterol and sitosterol into intestinal segments increased until 120 min after infusion of the micellar solution (Fig. 2A). After that, the uptake of cholesterol into intestinal segments tended to decrease, and that of sitosterol reached a plateau. There were no differences in uptake of cholesterol or sitosterol between the SHRSP and Wistar rats. The ratios of sitosterol to cholesterol were 0.1 to 0.2 at 5 and 10 min after dosage, and then they increased continuously until 240 min (Fig. 2B). No difference in the ratio was observed between SHRSP and Wistar rats.

Discussion

We have reported that depositions of campesterol and sitosterol were considerably higher in SHRSP than in Wistar rats.6 The present study indicates that lymphatic recovery of campesterol and sitosterol was comparable between the SHRSP and Wistar rats (Table 1). In contrast to these sterols, lymphatic recovery of stigmasterol and sitostanol in the SHRSP rats was higher than in the Wistar rats (Table 1). Since the amounts of these sterols absorbed were much lower than those of campesterol or sitosterol, the differences in amounts absorbed were very low, only $10.4 \mu g/100 g$ of body weight for stigmasterol and $11.0 \mu g/100 g$ of body weight for sitostanol for 24 h. We observed very low absorption and deposition of stigmastanol and sitostanol in the SHRSP rats.14 Low depositions of these sterols have also been observed in sitosterelemic patients15 and ABCG5/ABCG8 knockout mice.6 Our results suggest that acceleration of plant sterol absorption induced by the mutation of Abcg5 in SHRSP rats is rather small. Because the differences in the absorption of campesterol and sitosterol between the SHRSP and Wistar rats were small, there is a possibility that any differential absorption of these sterols was too small to observe. Our results also suggest that plant sterol deposition in the SHRSP rats, in comparison with the Wistar rats, is not caused by an increase in plant sterol absorption. Our second study found that plant sterol absorption in the SHRSP rats was not accelerated as compared with that in the Wistar rats. We measured the incorporation of radiolabeled sitosterol and cholesterol solubilized in the bile salt micelle into intestinal mucosal cells in the Wistar rats and the SHRSP rats (Fig. 2). Since radio-

Fig 2. Uptake of Cholesterol and Sitosterol in Jejunal Loops in Situ in SHRSP and Wistar Rats.
A micellar solution described in Materials and Methods, was prepared, and 0.5 ml of the micelle was administered to the jejunal loop in situ in SHRSP and Wistar rats. After incubation at 37°C for 5 to 240 min, the jejunal loop was resected and the levels of radioactivity in the unsaponifiable matter were measured. A, Time-dependent uptake of cholesterol and sitosterol into intestinal mucosa. Open circle, cholesterol uptake in Wistar rats; closed circle, cholesterol uptake in SHRSP; open square, sitosterol uptake in Wistar rats; closed square, sitosterol uptake in SHRSP rats. B, Ratio of sitosterol to cholesterol incorporated into intestinal mucosa. Open circle, ratio in Wistar rats; closed circle, ratio in SHRSP rats. Data are means ± SE of four to five rats per group.
labeled campesterol is not available, we used radio-labeled sitosterol in this study. If sterol efflux due to ABCG5 and ABCG8 in intestinal cells is impaired in SHRSP rats, the incorporation of these sterols into the cells must be higher in SHRSP than in Wistar rats. However, the incorporation of cholesterol and sitosterol into intestinal mucosal cells was almost the same between the SHRSP and the Wistar rats at all time points (Fig. 2A). These results mean that the mutation of Abcg5 did not influence the incorporation of sitosterol or cholesterol into the intestinal cells.

It has been reported that ABCG5 and ABCG8 are also expressed in the liver and are involved in the excretion of sterols into the bile. It is thought that the deposition of plant sterols in the body is determined by a balance between intestinal absorption and biliary excretion of the sterols. Hence, we think that expression of ABCG5 and ABCG8 in the intestine can be much smaller than in the liver. If so, deposition of plant sterols in the SHRSP rats as compared with the Wistar rats might have been caused by lower excretion into the bile. Our previous study indicated that SHRSP rats deposited more plant sterols in the liver than WKA and Wistar rats when these rats were fed a 0.5% plant sterol diet. In the study, biliary secretion of plant sterols was lower in the SHRSP rats than in the WKA rats. In ABCG5/ABCG8-deficient mice, biliary secretion of cholesterol almost disappeared.

Hence, we speculate that the ability to excrete plant sterols through ABCG5/ABCG8 is more important in the liver than in the intestine, and therefore, biliary secretion of plant sterols might be highly suppressed in SHRSP rats. We must measure differences in biliary excretion of plant sterols between SHRSP and Wistar rats in the future.

In contrast to our results in the present study, our previous observations indicated that lymphatic recovery of trace amounts of sitosterol was higher in SHRSP rats than in WKA rats. Batta et al. reported that WKY rats, with the same mutation in Abcg5 as SHRSP rats, showed higher absorption of trace amounts of orally dosed sitosterol than Wistar rats. We cannot explain the discrepancy between the results of this study and our previous observations. Trace amounts of radiolabeled sitosterol were administered in these studies.

In contrast, 6.7 mg/100 g of body weight of plant sterols was given in this study. In the previous studies, although the differences in radioactivity absorbed were significant, the amounts of the sterol absorbed were rather small. It is possible that differential absorption of plant sterols between SHRSP and Wistar rats can be observed only when they were given in small amounts. Further study is necessary to test this.

Igel et al. reported that when radiolabeled sitosterol and cholesterol were administered to the stomach of mice and periodical incorporation of the radioactivity into the intestinal mucosa was measured, the ratio of sitosterol to cholesterol was 0.75 at an early time (15 min), and rapidly decreased. They thought that since sitosterol was rapidly excreted from intestinal cells to the intestinal lumen, the ratios of sitosterol/cholesterol decreased. However, our present results do not support this. The ratio of sitosterol/cholesterol was lowest at around 0.1 to 0.2, at an early stage after dosage and then the ratio increased, probably because cholesterol is more rapidly incorporated into chylomicrons and secreted to the lymph than sitosterol (Fig. 2B). We have observed that absorbed sitosterol stayed in intestinal cells for a longer time than cholesterol, and released to the lymph more slowly. It has been reported that intestinal absorption of sitosterol is about 5% while that of cholesterol is about 50%. Therefore, the ratio of sitosterol to cholesterol at an early stage after dosage observed in this study agrees with the ratio of intestinal absorption. Our results do not support the possibility that sitosterol is rapidly incorporated into intestinal cells and then preferentially excreted to the intestinal lumen through ABCG5 and ABCG8.

The incorporated plant sterol and cholesterol at an early stage after administration were thought to be extremely low in quantity, because almost all of the suspension was assumed to be in the stomach. Hence, we infused a bile salt micelle containing sitosterol and cholesterol into the duodenum. Our experimental condition might be better to measure intestinal incorporation of sterols.

An important issue in the present study is the significant difference in body weight between the SHRSP and the Wistar rats. We administered plant sterols at a same amount per body weight in the SHRSP and the Wistar rats in experiment 1. However, it is not always true that our experimental condition is appropriate. More detailed studies are necessary in this respect.

It has been found that the incorporation of sterols through Niemann-pick C1-like 1 protein (NPC1L1) and simple diffusion is an important determinant in their intestinal absorption. We must evaluate the possibility that NPC1L1 and simple diffusion might compensate for malfunction of ABCG5/ABCG8 in SHRSP rats.

References


