Effects of Sitosterol and Sitostanol on Micellar Solubility of Cholesterol

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Summary The influence of sitosterol and sitostanol on the solubility of cholesterol in mixed bile salt micelles in vitro and in vivo was investigated to examine the mechanism by which sitostanol inhibits cholesterol absorption more than does sitosterol. Both sitosterol and sitostanol decreased micellar solubility of cholesterol to a similar extent, when determined with the turbidity. Also, these sterols reduced the concentration of cholesterol in micelles, both in vitro and in vivo. The extent of the reduction of micellar solubility of cholesterol by these sterols was almost the same in vitro, whereas sitostanol tended to reduce the solubility more effectively than sitosterol in vivo. Thus, the interference with cholesterol solubilization in vivo may be responsible for effective inhibition of cholesterol absorption by sitostanol. Since the effect of sitostanol was not observed in vitro, there is a possibility that another factor(s) not included in the in vitro system might affect the action of sitostanol on micellar solubility of cholesterol in vivo.

Key Words sitosterol, sitostanol, cholesterol absorption, mixed micelle, micellar solubility

Sitostanol, a hydrogenated product of sitosterol, is a more potent hypocholesterolemic agent than sitosterol in experimental animals and humans (1–3). We have shown that sitostanol inhibits cholesterol absorption more effectively than sitosterol in rats, although the mechanism is not defined clearly (1). Absorption of sitostanol is only 0 to 3% compared with around 10% of sitosterol in rats (1, 4–7). Less-absorbable sterols are known to inhibit cholesterol absorption (8, 9), and the effect generally depends on their physicochemical characteristics. We showed previously that the effect of less-absorbable sterols such as sitosterol and fucosterol on micellar solubility of cholesterol primarily determines the inhibitory potency to cholesterol absorption (10, 11). In addition, we suggested that the inhibitory effect of the sterols may also be influenced by the absorbability of these sterols per se (10),
and hence that the ability of any less-absorbable sterols to inhibit cholesterol absorption could be determined by measuring these parameters, i.e., absorbability of the sterols as well as the effect on micellar solubility of cholesterol.

In the present study, the effect of sitostanol on micellar solubility of cholesterol was compared with that of sitosterol \textit{in vitro} and \textit{in vivo}.

\section*{MATERIALS AND METHODS}

\subsection*{Chemicals.} Sitosterol was purchased from ICN Pharmaceuticals (Cleveland, Ohio) and was purified by recrystallizing several times from ethyl acetate and methanol to 99\% purity as sterol\textsuperscript{(12)}. The preparation contained 81\% sitosterol, 12\% sitostanol, 6\% campesterol, and 1\% campestanol. Sitostanol was prepared by hydrogenating sitosterol and purified as described previously\textsuperscript{(12)}. The preparation was composed of 93\% sitostanol and 7\% campestanol. Sodium taurocholate (purity \textgreater 96\%) was obtained from Calbiochem (La Jolla, Calif.). 1-Monooleoyl-rac-glycerol (purity 99\%), oleic acid (purity 99\%) and hydroxysteroid dehydrogenase, grade II, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Purified egg yolk lecithin containing 96\% lecithin and 3\% lysolecithin was kindly provided by QP Corp., Tokyo. Cholestanol was purchased from Tokyo Kasei Co. Tokyo.

\subsection*{Spectrophotometric and ultracentrifugal determination of micellar solubility of sterols.} Micellar solution contained 6.6 mM sodium taurocholate and 0.1 to 2.0 mM sterol (cholesterol, sitosterol, or sitostanol alone or in binary combinations) and, where indicated in the individual experiments, 0.6 mM egg yolk lecithin, 1 mM oleic acid, and 0.5 mM monoolein. These were prepared in 15 mM sodium phosphate buffer, pH 7.4, containing 132 mM NaCl by sonication.

Two milliliters of each micellar solution containing sterols singly or in binary combinations at 37°C were monitored immediately for turbidity by reading absorbance at 430 nm. The micellar solutions were maintained at 37°C overnight and observed for precipitates. In the separate study, micellar solution (5 ml) prepared as described above was ultracentrifuged for 1 h at a force of 100,000 \( \times g \) (Hitachi 55P-72, RP55T rotor) at 37°C. The concentration of each sterol in the clear supernatant was determined by mass measurement.

\subsection*{Animals and diets.} Male Sprague-Dawley rats (Seiwa Experimental Animals Co., Fukuoka) weighing 260–310 g were housed under a normal (12 h) light cycle and allowed laboratory chow (type NMF, Oriental Yeast Co., Tokyo) and water \textit{ad libitum} for 1 week. Rats were then meal-fed (10 am–11 am) a purified diet either with 0.5\% cholesterol, 0.5\% cholesterol plus 0.5\% sitosterol, or 0.5\% cholesterol plus 0.5\% sitostanol for 10 days. The diets were prepared according to guidelines recommended by the American Institute of Nutrition\textsuperscript{(13)} and contained by weight (\%) casein, 20; safflower oil, 10; AIN-76\textsuperscript{TM} mineral mixture, 3.5; AIN-76\textsuperscript{TM} vitamin mixture, 1; choline bitartrate, 0.2; cellulose powder, 5; methionine, 0.3; corn starch, 15; and sucrose to 100. Cholesterol, sitosterol, and sitostanol were added at the expense of sucrose. AIN-76\textsuperscript{TM} mineral and vitamin mixtures were obtained from J. Nutr. Sci. Vitaminol.
Nihon Nosan Kogyo Co., Kanagawa. Two hours after withdrawal of the diet, rats were killed by decapitation. The intestine and liver were quickly excised and the intestinal contents were collected as described (10). After inactivation of enzymes at 70°C for 15 min, the contents were ultracentrifuged and the clear supernatant and the precipitate were collected as described above.

**Steroid analyses.** Lipids in micelles and intestinal contents were extracted with chloroform: methanol (2:1, v/v) according to Folch et al. (14). The purified lipids were saponified with ethanolic KOH and unsaponifiable matters were collected with petroleum ether. Sterols were transformed to trimethylsilyl derivatives and analyzed on a Shimadzu gas chromatograph GC-4CMPF with a flame ionization detector and a glass column packed with GasChrome Q, 60–80 mesh, coated with 3% OV-17 (Gasukuro Kogyo Inc., Tokyo). Cholestan (Nacalai Tesque Inc., Kyoto) was used as an internal standard.

Bromine-chloroform solution (3.1 µg/ml) for bromination was added to sitosterol and sitostanol preparations. The solution was dried up under nitrogen, and after the addition of ethanol–acetone solution (1:1, v/v), stanol was precipitated with 0.5% digitonin in 50% ethanol and kept overnight. Stanol digitonide was washed with ethylether and dissolved in dimethyl sulfoxide. Stanol released was extracted with hexane and converted to the trimethylsilylether. Stanol was analyzed on a Shimadzu GC-9A gas chromatograph with a flame ionization detector and a fused silica capillary column (25 m × 0.24 mm) coated with silicone OV-1 (Chromato Packing Center, Kyoto). Small amounts of sterol (1% of original sterol) which were not brominated were separated with OV-1 capillary column. Recrystallized cholestanol was used as an internal standard. Bile acids were measured enzymatically with hydroxysteroid dehydrogenase according to the method of Eaton and Klaassen (15).

**Statistical analysis.** One-way analysis of variance was used to determine significant difference in the effects of dietary sterols. The exact nature of the difference among each group was determined by Duncan’s multiple-range test.

**RESULTS**

**Micellar solubility of cholesterol, sitosterol, sitostanol, and in binary combinations in vitro: Qualitative estimation**

Sets of micellar solubility curves measured as turbidity are shown in Figs. 1 to 3. Three kinds of micellar solutions composed of bile salt-lecithin (system 1), bile salt-monoolein-oleic acid (system 2), and bile salt-lecithin-monoolein-oleic acid (system 3) were prepared as described in the MATERIALS AND METHODS section. Micellar solution containing negligible or low concentrations of sterols was clear with an absorbance of less than 0.03 in any micellar system. As the concentration of sterol increased and exceeded the concentration beyond the solubilizing capacity of the micelles, the micellar solution became turbid. Micellar solubility was the highest in cholesterol, the lowest in sitostanol or sitosterol, and intermediate in binary
Fig. 1. Micellar solubility of sterols or their binary mixtures in system I in vitro. Cholesterol (C, ▲), sitosterol (S, ○), sitostanol (HS, □), C+S (●), and C+HS (■) in equimolar mixtures containing 0.1 to 2.0 mM total sterol were sonicated at 37°C in 2.0 ml of 15 mM sodium phosphate buffer, pH 7.4, containing 6.6 mM sodium taurocholate, 0.6 mM egg yolk lecithin, and 132 mM sodium chloride. Turbidity was measured spectrophotometrically at 430 nm. The results are the mean for n=2. * Precipitates were observed.

Fig. 2. Micellar solubility of sterols or their binary mixtures in system II in vitro. See Fig. 1 legend. Micellar solution contained 1 mM oleic acid and 0.5 mM monoolein instead of 0.6 mM egg yolk lecithin. The results are the mean for n=2. * Precipitates were observed.

mixture of cholesterol and either sitosterol or sitostanol in all the micellar systems examined. However, the effect of sitostanol on micellar solubility of cholesterol was not different from that of sitosterol in any micellar systems.
Fig. 3. Micellar solubility of sterols or their binary mixtures in system III in vitro. See Figs. 1 and 2 legends. Micellar solution contained egg yolk lecithin, oleic acid, and monoolein at the same concentration as in Figs. 1 and 2. The results are the mean for \( n = 2 \). * Precipitates were observed.

Fig. 4. Micellar solubility of cholesterol or its binary mixtures with sitosterol or sitostanol in vitro. Micellar solutions (5 ml) were prepared in system II (A) and system III (B) as in the case of Figs. 1 to 3. Micellar solutions were prepared with 1 and 2 mM cholesterol (C) and with 1 mM cholesterol plus 1 mM sitosterol (C+S) or sitostanol (C+HS). The solution was ultracentrifuged at 100,000 \( \times g \) for 60 min at 37°C. The supernatant was collected, and concentrations of cholesterol, sitosterol, and sitostanol were determined by GLC. ■, cholesterol; □, sitosterol; ▼, sitostanol. The results are shown as the mean ± SE, \( n = 4 \).

**Effects of sitosterol and sitostanol on micellar solubility of cholesterol in vitro: Quantitative estimation**

For quantitative estimation of the effect of sitosterol and sitostanol on micellar solubility of cholesterol in vitro, micellar solutions of systems II and III containing 1 and 2 mM cholesterol alone or 1 mM cholesterol plus 1 mM either sitosterol or sitostanol were ultracentrifuged and the micellar concentration of each sterol was measured in the clear supernatants. As shown in Fig. 4, A and B, sitosterol or
sitostanol lowered micellar solubility of cholesterol. However, no different effect of sitosterol and sitostanol was observed in this experimental condition.

**Effect of sitosterol and sitostanol on micellar solubility of cholesterol in vivo**

Rats were meal-fed for 10 days a diet containing either 0.5% cholesterol, 0.5% cholesterol plus 0.5% sitosterol, or 0.5% cholesterol plus 0.5% sitostanol, and 2 h after the final meal they were killed and intestinal aqueous micellar phase was collected. Body weight, food intake, and liver weight were comparable among the three groups (data not shown). Liver cholesterol was significantly decreased by sitosterol and sitostanol (Table 1), showing the inhibitory effect of these sterols on cholesterol absorption. Volumes of the aqueous phase and the bile acid concentration in intestinal micellar aqueous solution were comparable among the groups. The concentration of cholesterol in micellar solution was extremely low as compared with that in the solid phase which was collected after the removal of the micellar phase. Solubility of cholesterol in the micellar aqueous phase of rats fed cholesterol plus sitostanol and cholesterol plus sitosterol was 47% and 76% that in rats fed cholesterol alone. However, the difference was not significant because of a large variation. Solubility of sitostanol in micellar solution tended to be low compared with that of sitosterol. The content of cholesterol in the solid phase in rats fed cholesterol plus sitosterol was comparable with that in rats fed cholesterol plus sitostanol. However, it was markedly higher than that in the group fed cholesterol

| Groups       | Liver cholesterol (mg/g) | Aqueous phase | Solid phase | Aqueous phase |
|--------------|--------------------------|---------------|-------------|---------------|
|              |                          | Cholesterol  | Sitosterol* | Cholesterol  | Sitosterol* |
|              |                          | (µg)         | (µg)        | (mg)         | (mg)        |
| C            | 25.6 ± 2.1*a             | 517 ± 111    | 52.0 ± 12.5*a| 7.80 ± 0.71*a| 0.94 ± 0.09*a| 41.2 ± 5.7   |
| C+S          | 7.81 ± 1.45              | 391 ± 67     | 357 ± 68    | 10.9 ± 0.6   | 14.6 ± 0.7   | 36.0 ± 4.7   |
| C+HS         | 8.76 ± 0.71              | 244 ± 43     | 232 ± 45    | 11.4 ± 0.9   | 14.9 ± 1.3   | 36.3 ± 5.1   |

*Significantly different from both C+S and C+HS groups at $p < 0.01$. *This fraction also contained sitostanol.

Table 1. Effect of sitosterol and sitostanol on the micellar solubility of cholesterol in intestinal luminal contents.

Rats that were meal-fed a semi-purified diet containing 0.5% cholesterol alone (C) or plus 0.5% sitosterol (C+S) or plus 0.5% sitostanol (C+HS) for 10 days were killed 2 h after they consumed the final meal. Luminal content was collected, heated to inactivate enzymes, and centrifuged at 100,000 $\times g$ for 60 min. Sterol concentrations in aqueous and solid phases were determined by GLC. Volumes of the aqueous phase were $1.1 ± 0.2$ ml, $1.3 ± 0.2$ ml, and $1.2 ± 0.3$ ml for group C, C+S, and C+HS, respectively. The results are shown as mean ± SE, $n=6$ or $7$.
alone. There was no difference in the content of both sitosterol and sitostanol in the solid phase.

DISCUSSION

The absorption of cholesterol by the intestinal tract involves several complex processes, which have been reviewed elsewhere (16, 17). The major steps include: i) solubilization of unesterified cholesterol in mixed micelles composed of bile salts, phospholipids, and products of triglyceride digestion, ii) transport of cholesterol through mucosal barriers such as unstirred water layer and mucoprotein, iii) incorporation of cholesterol into brush border membranes of intestinal absorptive cells, iv) intracellular transport of cholesterol to the site of esterification, v) esterification of cholesterol, and vi) incorporation of cholesterol into lipoproteins and the release to lymph.

Several hypotheses on the mechanisms of the inhibition of cholesterol absorption by sitosterol were proposed in classical studies without any experimental evidence (6, 16). These included i) formation of a nonabsorbable complex with cholesterol in the intestinal lumen (18), ii) competitive inhibition of cholesterol uptake by intestinal mucosal cells (19), and iii) interference with cholesterol esterification in mucosal cells (20). Series of our systematic studies (10, 11) revealed that sitosterol decreased intestinal micellar solubility of cholesterol in binary mixtures in vitro and in vivo. In contrast, fucosterol, which is a less effective inhibitor of cholesterol absorption than sitosterol, solubilized more cholesterol in micelles than sitosterol. Competitive inhibition of cholesterol uptake from brush border membranes and the inhibition of cholesterol esterase and acyl-CoA cholesterol acyltransferase (ACAT) in the intestinal mucosa were not observed in our studies. No effect of sitosterol on cholesterol esterification by ACAT was also supported in a critical study (21).

Many other sterols are known to interfere with cholesterol absorption. However, it is not defined whether the same mechanism as in the case of sitosterol and fucosterol functions in the inhibitory effect. The results of this study showed that sitostanol, which inhibits cholesterol absorption more efficiently than sitosterol (1), reduced micellar solubility of cholesterol in vivo to a greater extent than sitosterol. Thus, cholesterol solubility in aqueous micellar solution in rats fed cholesterol plus sitostanol was 53% lower than that in rats fed cholesterol alone, compared with 24% in rats fed cholesterol plus sitosterol. However, the effect in vivo was not reproduced in vitro. Many factors including dietary and bile components as well as several lipolytic enzymes participate in the complex processes of micelle formation in the intestinal lumen. Therefore, it seems difficult to reproduce the in vivo condition in the in vitro system. We used taurocholic acid as only one bile acid to micellarize sterols. Since different bile salts have different capacities of micellar solubilization of sterols, more detailed study in vitro using a bile salts mixture similar to the bile may give us precise information. The fatty acid composition of
dietary fat, which is another important factor influencing micellar solubility of cholesterol, was unlikely to modify the differential effect of sitosterol and sitostanol, because the superior effect of sitostanol to sitosterol on fecal cholesterol excretion was not altered with different sources of dietary fat (1).

In the previous study regarding the inhibitory effect of less-absorbable sterols on cholesterol absorption, we proposed that the absorbability of the sterols determines the potency of the inhibitory effect; the less they are absorbed, the more they become effective to displace cholesterol from micelles by remaining in the intestinal lumen (10). The present results, however, did not support this hypothesis. Sitostanol rather decreased total amounts of sterols solubilized in the micellar phase in vivo.

Several investigations showed that sitosterol exerted a cholesterol-lowering activity in experimental animals after it was absorbed (6). However, this is not the case for sitostanol, because its absorption is almost negligible (1, 4).

The observation that micellar solubility of cholesterol reduced more efficiently when it was combined with sitostanol than when combined with sitosterol in vivo may be one of the mechanisms by which sitostanol effectively inhibits cholesterol absorption. However, the results of the micellization study in vitro did not support the predominant effect of sitostanol, suggesting that the micellar systems constructed in this experiment in vitro do not necessarily represent those in vivo.

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