INHIBITION OF β -SITOSTEROL ON INTESTINAL CHOLESTEROL ABSORPTION IN RAT USING IN VIVO DUAL ISOTOPE RATIO METHOD

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Summary The inhibitory effects of β -sitosterol on intestinal cholesterol absorption were studied by means of a dual isotope plasma ratio method (*in vivo*), which is a new technique for the measurement of cholesterol absorption, as well as a ligated-loop method (*in situ*). The results obtained were as follows:

1. The absorption of β -sitosterol itself was significantly less than cholesterol. Cholesterol was selectively absorbed from rat intestine.
2. When 100 to 1,000 μ g of β -sitosterol were added to the dose solution containing 10 μ g of cholesterol, cholesterol absorption by the *in vivo* experiment decreased with the increase of additional β -sitosterol.
3. A similar inhibitory effect of β -sitosterol was observed by the *in situ* ligated-loop method.

These results suggest that β -sitosterol actually inhibits cholesterol absorption in the physiological state of an animal.

Keywords dual isotope, ligated-loop, selective absorption, β -sitosterol, cholesterol, intestinal (absorption), inhibition

Some plant sterols, such as β -sitosterol and stigmasterol, are thought of as agents preventing hypercholesterolemia. The lowering effect of dietary β -sitosterol on serum cholesterol of rat (1), chick (2) and man (3, 4) suggest that β -sitosterol may inhibit the intestinal absorption of cholesterol. Mattson *et al.* (5) originally showed the effect of β -sitosterol in rats with lymph-duct fistula, which gave a direct estimation of cholesterol absorption from the intestinal tract. However, Sylvén and Borgström demonstrated contradictory results on intestinal absorption of cholesterol and β -sitosterol (6) using the same methods. It is not yet clear whether the

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inhibitory effect of β -sitosterol would actually occur on the intestinal absorbing process of cholesterol or not.

Recently, Zilversmit *et al.* have developed an *in vivo* dual isotope plasma ratio method as a new technique for the measurement of cholesterol absorption in rat (7,8). The method has been evaluated as very reliable and useful in the physiological state of animals, and even on man (9). Therefore, it seemed interesting to investigate the effect of β -sitosterol on cholesterol absorption using the dual isotope ratio method.

The present paper describes the β -sitosterol effect on intestinal cholesterol absorption by not only the *in vivo* dual isotope ratio method but the *in situ* ligated-loop technique.

METHODS

1. *Chemicals and animals.* 7- ^3H -Cholesterol (s.a. 21.5 Ci/mmol) was obtained from New England Nuclear Co. (Boston, MA), and 4- ^{14}C -cholesterol (s.a. 0.057 Ci/mmol), 22,23- ^3H - β -sitosterol (s.a. 58 Ci/mmol) and 4- ^{14}C - β -sitosterol (0.053 Ci/mmol) were obtained from Amersham/Searle Co. (Arlington Height, IL). The purity of these radioactive sterols was tested by thin layer chromatography on silica gel G (Merck Co., Darmstadt, Germany) with the solvent system of benzene:ethylacetate = 2:1 (v/v). Sterols with a purity of more than 93% were used in all experiments. The micellar dose solutions containing radioactive cholesterol (1.0 $\mu\text{Ci}/0.5\text{ ml}$) or β -sitosterol (1.0 $\mu\text{Ci}/0.5\text{ ml}$) were prepared by dissolving various amounts of the corresponding non-radioactive sterol and 5 mM glycerol mono-oleate (Sigma Chem. Co., St. Louis, MO) in 50 mM sodium phosphate buffer (pH 6.3) containing 5 mM glycochenodeoxycholic acid (Tokyo Tanabe Co., Tokyo). These solutions were incubated at 37°C overnight to produce stable micellar particles.

Sprague-Dawley rats weighing 250–300 g were fed with a commercial chow (Oriental Yeast Co., Tokyo) *ad libitum*, and fasted for 24 hr before the experiments.

2. *Dual isotope plasma ratio method (in vivo).* The measurement of cholesterol and β -sitosterol absorption from the intestine were carried out by the dual isotope ratio method (8,9) with slight modifications as follows; under light anesthesia with ether, the abdominal cavity was opened by incision, and 1.0 μCi of ^{14}C -cholesterol or ^{14}C - β -sitosterol in 0.5 ml of micellar dose solution was administered into the duodenum. Immediately after the dosage, 1.0 μCi of ^3H -cholesterol or ^3H - β -sitosterol dispersed in 0.5 ml of serum obtained previously from the same animal was also injected into the left external jugular vein (I.V. dose). Thereafter, the rat was fed with the same diet and water *ad libitum* in a wire cage, and 2 ml of blood were collected from the right external jugular vein of the rat with heparinized syringes at 48, 72 and 96 hr. The radioactive sterols were extracted from the plasma samples (1 ml each) with 3 ml of ethanol:hexane = 2:1 (v/v) after saponification with 50% KOH at 50°C for 30 min. Both radioactivities of ^3H - and

[^{14}C]-sterol were then determined simultaneously in a toluene-based scintillation cocktail containing 0.4% Omnifluor (New England Nuclear Co., Boston, MA) by a Packard Tri-Carb liquid scintillation spectrometer, Model 3380 (Packard Co., Downers Grove, IL). Percent absorption of cholesterol or β -sitosterol was calculated by the formula as follows:

$$\% \text{ absorption} = \frac{[\text{plasma } [^{14}\text{C}] \text{ (DPM)}] / [\text{dosed } [^{14}\text{C}] \text{ (DPM)}]}{[\text{plasma } [^3\text{H}] \text{ (DPM)}] / [\text{dosed } [^3\text{H}] \text{ (DPM)}]} \times 100$$

3. *Ligated-loop method (in situ)*. The measurements for intestinal mucosal uptake of sterols were carried out by the (*in situ*) ligated-loop method of Sylvén (10). After an animal was anesthetized with nembutal (30 mg/kg), 4 to 6 segments of 4-cm length were made in the jejunum of an animal with an intact current of blood and lymph. One-hundredth μCi of [^{14}C]-cholesterol or [^3H]- β -sitosterol in 0.5 ml of dose solution was injected into each segment, and then incubated in the body for 30 min after the abdominal wall had been closed. At the end of the incubation, each segment of the loop was washed with 2 ml of ice-chilled physiological saline. Then, the tissue was further washed 5 times with 2 ml of ice-chilled 5 mM sodium deoxycholate in saline to remove the sterols that remained on the surface of the mucosa. Finally, each washed tissue was weighed (200–400 mg), then the radioactive sterols were extracted with 9 ml of chloroform:methanol = 2:1 (v/v), as described by Folch *et al.* (11). After the solvent had dried, the radioactivities were determined, and the amount of sterol incorporated into the tissue was calculated and expressed as $\mu\text{g}/100\text{ mg tissue}/30\text{ min}$.

RESULTS

1. Selective absorption of cholesterol and β -sitosterol

β -Sitosterol absorption was measured by the dual isotope ratio method, since different results on intestinal absorption of β -sitosterol have been reported by some laboratories using other methods such as a balance study (12, 13) and lymph collection (6). The absorption ratio of β -sitosterol was $5.4 \pm 1.6\%$ (in 500 μg dose) and that of cholesterol was $44.2 \pm 3.8\%$ (in 500 μg dose) (Table 1). These results show that β -sitosterol is resistant to intestinal absorption, and cholesterol is selectively absorbed from rat intestine.

2. Effect of β -sitosterol on the absorption and mucosal uptake of cholesterol

In order to observe the β -sitosterol effect on cholesterol absorption, 100 to 1,000 μg of β -sitosterol (Gaskuro Co., Tokyo, purity 98%) were added to 10 μg of [^{14}C]-cholesterol (1.0 μCi) in a 0.5 ml dose solution, and cholesterol absorption was measured by the dual isotope ratio method. As shown at left in Fig. 1, cholesterol absorption clearly decreased with an increase of additional β -sitosterol. The absorption ratio of cholesterol was about 48.3% of control in the presence of

Table 1. The selective absorption of cholesterol and β -sitosterol.

	Cholesterol	β -Sitosterol
DIRM (<i>in vivo</i>)	44.2 ± 3.8^a	5.4 ± 1.6^a
Ligated-loop (<i>in situ</i>)	10.3 ± 2.7^b	3.5 ± 0.7^b

Five hundred μg of [^{14}C]-cholesterol or [^{14}C]- β -sitosterol in a 0.5 ml dose solution were given into the duodenum and % absorption of sterol was measured by DIRM as described elsewhere. In the *in situ* ligated-loop method, 100 μg of [^{14}C]-cholesterol or [^3H]- β -sitosterol in a 0.5 ml of dose solution were injected into the jejunal segment and the amount of sterol incorporated in mucosa was measured. The values were expressed as μg of sterol per 100 mg tissue per 30 min incubation.

^a % absorption per rat. ^b μg of sterol incorporated per 100 mg tissue per 30 min.

Fig. 1. Inhibitory effect of β -sitosterol on cholesterol absorption and mucosal uptake. The cholesterol absorption ratio in the administration of 10 μg of cholesterol in a 0.5 ml dose solution was measured by DIRM in the presence of 0 (control), 100, 500 and 1,000 μg of β -sitosterol (left in Fig. 1). The values are expressed as % of control. Cholesterol uptake into the jejunal mucosa of a segment was measured by the *in situ* ligated-loop method in the presence of 0 (control), 10, 100, 200 and 500 μg of β -sitosterol (right in Fig. 1). Cholesterol given into a segment was 10 μg in a 0.5 ml dose solution. The values (μg of incorporated cholesterol/100 mg tissue/30 min) were also expressed as % of control.

1,000 μg of β -sitosterol (100 times the amount of cholesterol). The β -sitosterol effect on cholesterol absorption was also studied by the ligated-loop technique.

When 10 to 500 μ g of β -sitosterol were added to the dose solution containing 10 μ g of cholesterol, the mucosal uptake of cholesterol decreased with an increase of additional β -sitosterol (right in Fig. 1). The cholesterol uptake was about 36% of the control in the presence of 500 μ g of β -sitosterol (50 times the amount of cholesterol).

These results strongly indicate that β -sitosterol has an inhibitory effect on intestinal cholesterol absorption. Furthermore, the results support that both the methods, *in vivo* dual isotope ratio method and *in situ* ligated-loop method, bring similar results of intestinal absorption and mucosal uptake of cholesterol.

DISCUSSION

Sylvén and Borgström(6) have demonstrated that β -sitosterol showed no inhibitory effect on cholesterol absorption in rat with lymph duct fistulae. However, the result seems to occur in animals in which cholesterol metabolism might lead to an unsteady state during the long-term collection of lymph. Actually, the absolute value of absorbed cholesterol in their experiment was much lower than that of other groups(5, 14). On the other hand, Mattson *et al.*(5) showed the inhibitory effect of β -sitosterol in rats with lymph duct fistulae when an excess amount of β -sitosterol (more than 30 mg per rat) was administered. The inhibitory effect of β -sitosterol observed in their experiment may be related to the reduction in cholesterol solubility to disturb the micell formation in a stage before absorption. However, our results also indicated that cholesterol absorption and mucosal uptake were decreased in the presence of β -sitosterol, in spite of the total sterol concentration being much lower (3 mM or less in 5 mM bile acid) as Borgström has reported(15). In our results, β -sitosterol seems to have other inhibitory mechanisms than the reduction in cholesterol solubility on intestinal cholesterol absorption. Furthermore, similar inhibitory effects of β -sitosterol observed by both the *in vivo* dual isotope ratio method and *in situ* ligated-loop technique appears to suggest that the inhibition takes place at the stage of mucosal uptake in the absorption process of cholesterol. As β -sitosterol actually led to a decrease in cholesterol uptake in the *in situ* experiment, while the cholesterol incorporated into mucosa was not detected in the lymph or blood during the 30-min incubation. The selective absorption of sterols would also be related to the stage of mucosal uptake as suggested elsewhere(16).

From the present study, it was demonstrated that β -sitosterol inhibited the absorption of cholesterol in the physiological state. However, further observations are necessary to clarify the mechanism of the inhibitory effect of β -sitosterol on cholesterol absorption.

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