**Note**

**Serum Cholesterol Reduction and Cholesterol Absorption Inhibition in CaCo-2 Cells by a Soyprotein Peptidic Hydrolyzate**

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The serum cholesterol level in rats was significantly decreased in a group fed on a soyprotein peptic hydrolyzate (SPH) when compared with a group fed on a casein tryptic hydrolyzate (CTH). The fecal excretion of total steroids was significantly greater with rats fed on the SPH diet when compared with the CTH diet. The results of CaCo-2 studies clearly suggest that the suppression of cholesterol absorption in the intestinal epithelia is part of the mechanism for the hypocholesterolemic action induced by SPH.

**Key words:** cholesterol; soyprotein; casein; CaCo-2; intestine

Several reports have indicated that the quality and quantity of dietary protein affect the serum cholesterol level. 1–9) Soyprotein, a vegetable protein, is well known to reduce serum cholesterol, in comparison with the effect of casein, an animal protein. In addition, a soyprotein peptic hydrolyzate (SPH) has been reported to have a stronger lowering effect on serum cholesterol than that by intact soyprotein. 10,11) The fact that the ingestion of soyprotein or SPH increased the fecal excretion of steroids enables it to be indirectly inferred that each would exert a lowering effect on serum cholesterol by inhibiting the absorption of cholesterol from the intestinal tract. 10,11) However, no direct measurements on cholesterol absorption from the intestines have been reported with SPH. It therefore remains unclear which peptides derived from SPH could be involved in the inhibition of cholesterol absorption from the small intestine, and nothing is known about the molecular mechanism for its inhibitory effect on absorption. This has made it extremely difficult to identify the active component in vivo, after animals like rats have been fed with SPH not only because it is necessary to prepare a large amount of SPH but also because SPH contains several kinds of peptide. No work has so far been reported to evaluate the effects of such trace peptides as SPH, on the absorption of cholesterol in vitro. We report in this paper an experimental system to evaluate the effects of a soyprotein peptic hydrolyzate (SPH) and casein tryptic hydrolyzate (CTH) on cholesterol absorption by using CaCo-2 cells which expressed the function of epithelial cells in the small intestine. We postulate that a SPH-induced hypocholesterolemic action may have brought about an inhibition of cholesterol absorption in the intestinal epithelial cells. We therefore used this cell culture system to investigate the serum cholesterol-lowering action of SPH.

Male rats of the Wistar strain (Japan SLC Inc., Hamamatsu, Japan) weighing about 90 g were used in Experiment 1. The room temperature was maintained at 22 ± 2°C with a 12-h light (08:00–20:00) and dark cycle. All the rats were individually housed and provided with food and water *ad libitum*. The rats were fed on a commercial stock diet (CE-2; Japan CLEA Co., Ltd., Tokyo) for 3 days to allow them to adapt to the new environment, after which they were fed on a purified diet containing either a casein tryptic hydrolyzate (Meiji Milk Products Co., Ltd., Tokyo, Japan) or a soybean protein peptic hydrolyzate (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) for 10 days. The purity was 86.2% and 81.9% for the casein and soybean hydrolyzates, respectively. The composition of the basal diet was according to the formula recommended by American Institute of Nutrition, 12) in weight percent as follows: soybean protein peptic hydrolyzate or casein tryptic hydrolyzate, 20; lard, 5; corn oil, 1; AIN76 mineral mixture, 3.5; AIN76 vitamin mixture, 1.0; choline bitartrate, 0.2; cellulose, 5; corn starch 42.03; sucrose 21.02; cholesterol, 0.5; sodium cholate, 0.25; 0.23% or 0.15% DL-methionine was added to the diets containing the soyprotein hydrolyzate or casein tryptic hydrolyzate, respectively. After 7–9 days on each diet, feces were collected from the rats and used for determining fecal steroids. At the end of the experimental period, the rats were anesthetized with diethyl ether and killed at 09:00 hours. Blood was collected by cardiac puncture for an analysis of serum cholesterol, and the liver of each rat was immediately excised for an analysis of liver lipids.

Serum and liver cholesterol levels were measured by an enzymatic colorimetric method (Monostest Cholesterol; Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan). The serum concentration of high-density lipoprotein (HDL)-cholesterol was assayed with a commercially available kit (Wako Pure chemical Ind., Ltd., Osaka, Japan). Liver lipids were extracted by the method of Folch et al. 13) and total lipids were determined gravimetrically as described previously. 14) Fecal acidic steroids were measured according to the method of Bruusgaard et al.15) and Malchow-Moller et al. 16) while fecal neutral steroids were assayed as the trimethyl silyl ether by using 1.5% OV-17 with a GC-14A instrument (Shimadzu, Kyoto, Japan) and 5α-cholestan as an internal standard. 17)

CaCo-2 cells were a generously presented by Central Research Institute of Meiji Milk Products Co., Ltd. (Tokyo, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4 mM l-glutamine, 50 IU/ml of penicillin and 50 μg/ml of streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The monolayers became confluent 3 to 4 days after seeding at between 7 × 104 and 1.2 × 105 cells per 100-mm diameter dish, and the cells were passaged at a split ratio of 4 to 8 by trypsinizing with 0.25% trypsin and 0.8 mM disodium ethylenediamine tetraacetate in phosphate-buffered saline (PBS). Monolayers were grown in 60-mm plastic Petri dishes containing 5 ml of supplemented DMEM as described previously, 18) fresh medium being added every 2 days. The experiments described usually used cultures 11–13 days after plating, the experiments

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being performed in medium-199/Earle's containing 1 mM HEPES. Cell viability, as ascertained by trypan blue exclusion, was unaffected by any of the experimental procedures. The number of passages of the cell line ranged from 40 to 50.

Stock solutions of cholesterol, monoolein, and phosphatidylcholine were prepared in chloroform, while a stock solution of sodium taurocholate was made in 95% ethanol. The lipids and bile salt were mixed, and the solvents were evaporated under a stream of nitrogen. Medium-199 with HEPES was then added so that the final concentration of each [14C]-labelled micellar solution was as follows: [14C]-cholesterol (NEN). 57.5 mCi/mmol, 0.02 μg; monolein, 1 mm; taurocholic acid, 5 mm; soyprotein peptic hydrolyzate (SPH), 1 mg/ml or 10 mg/ml; casein trypsin hydrolyzate (CTH), 1 mg/ml or 10 mg/ml. The micellar solution was mixed by ultrasonic vibration for 3 min (25 W). CaCo-2 cells were grown in 60-mm plastic Petri dishes to investigate the effects of SPH or CTH on cholesterol absorption. After 14 days, the cells were rinsed two times with 5 ml of Dulbecco's phosphate-buffered saline (PBS). A [14C]-labelled micellar solution (5 ml) containing SPH or CTH was then added to the dishes which were incubated at 37°C for 20 min in a CO2 incubator. After this incubation, the cells were rinsed two times with 5 ml of PBS. The cells were finally lysed in a 0.1% SDS solution, before 7.5 ml of Aquasol (Dupont) was added, and the radioactivity in the cellular debris was counted to determine the amount of cholesterol associated with the cells.

The statistical significance of differences between values was analyzed by Student's t-test.10

Food intake and growth rate were unaffected by the protein hydrolyzate source, but the relative liver weight was significantly less in the soyprotein peptic hydrolyzate (SPH) group. Serum total cholesterol concentration was significantly less in the SPH-fed group than in the casein trypsin hydrolyzate (CTH) group. The level of HDL-cholesterol was unaffected by the protein hydrolyzate source, and most of the decrease in serum cholesterol was associated with LDL + VLDL-cholesterol. The proportion of HDL-cholesterol to serum total cholesterol [(b)/a)] was significantly higher in the SPH-fed group. The liver total lipids and cholesterol levels were significantly lower in the SPH-fed group than in the CTH-fed group, while fecal dry weight was significantly higher in the SPH-fed group than in the CTH-fed group. The output of acidic steroids and cholesterol in the feces was unaffected by the protein hydrolyzate source. The fecal excretion of coprostanol was significantly more in the SPH-fed group than in the CTH-fed group, as was the fecal excretion of total steroids (acidic steroids + neutral steroids).

We confirmed that the CaCo-2 cells expressed alkaline phosphatase and succrase as a marker enzyme of small intestinal epithelial cells (data not shown in the table). The cholesterol uptake in CaCo-2 cells was decreased significantly by adding SPH (1 mg/ml or 10 mg/ml) when compared with the effect of CTH.

Sugano et al.16,17 have shown that a soyprotein peptic hydrolyzate (SPH) both decreased the blood cholesterol level and promoted fecal excretion of steroids, as compared with the effect of casein. They suggested that SPH might have inhibited cholesterol absorption more than casein did from the results of fecal steroids excretion. We report that SPH caused both a decrease in serum cholesterol and an increase in the fecal excretion of steroids, as compared with the casein hydrolyzate prepared from casein by trypsin. The present results are similar to those of Sugano et al.10,11 and suggest that SPH could not only decrease blood cholesterol but also inhibit cholesterol absorption. Although the soyprotein effect on cholesterol absorption has previously been examined in comparison with that by casein,20 the in vivo experimental system did not allow its direct effect on cholesterol absorption. Until now, there has been no direct evidence that SPH would inhibit cholesterol absorption in the intestine. Moreover, Saeki et al.21 have suggested that the inhibition of cholesterol absorption was not the major factor involved in the differential effects of dietary proteins on serum cholesterol. Therefore, to elucidate the molecular mechanism for the inhibitory effect of SPH on cholesterol absorption, we constructed an in vitro experimental system to evaluate the effect of SPH on cholesterol absorption by using an in vitro-cultured cell strain.

In recent studies, monolayers of CaCo-2 cells cultures that had been isolated from a colon carcinoma have been used as a model system to examine the process of lipid metabolism.22-28 Despite their colonic origin, CaCo-2 cells spontaneously differentiated into polarized, columnar cells that showed many morphological and physiological characteristics of mature enterocytes in the small intestine. For instance, CaCo-2 cells which developed the function of epithelial cells in the small intestine have been used to elucidate the molecular mechanism for cholesterol absorption. Field et al.24 have reported that CaCo-2 cells, like the small intestine, had the ability to absorb micellar cholesterol and to express marker enzymes like alkaline phosphatase as small intes-

### Table I. Effects of a Dietary Casein Tryptic Hydrolyzate (CTH) and Soyprotein Peptic Hydrolyzate (SPH) on Body and Liver Weights, Food Intake, Serum and Liver Lipids, and Fecal Steroid Excretion in Rats*

<table>
<thead>
<tr>
<th>Measure</th>
<th>CTH</th>
<th>SPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/10 days)</td>
<td>40.4 ± 2.7</td>
<td>48.6 ± 2.6</td>
</tr>
<tr>
<td>Food intake, 10 days (g/d)</td>
<td>12.2 ± 0.5</td>
<td>13.7 ± 0.6</td>
</tr>
<tr>
<td>Liver (% body wt.)</td>
<td>4.90 ± 0.15</td>
<td>4.26 ± 0.10</td>
</tr>
<tr>
<td>Serum (mg/100 ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (a)</td>
<td>230 ± 10</td>
<td>104 ± 9</td>
</tr>
<tr>
<td>HDL-cholesterol (b)</td>
<td>48.9 ± 4.1</td>
<td>45.1 ± 2.3</td>
</tr>
<tr>
<td>LDL + VLDL-cholesterol (b/a)</td>
<td>181 ± 10</td>
<td>59.2 ± 7.4</td>
</tr>
<tr>
<td>(b/a)</td>
<td>0.21 ± 0.02</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>Liver (mg/g wet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total lipids</td>
<td>103 ± 4</td>
<td>72.8 ± 1.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20.1 ± 0.9</td>
<td>11.0 ± 0.6</td>
</tr>
<tr>
<td>Fecal excretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry weight (g/3d)</td>
<td>3.07 ± 0.10</td>
<td>4.22 ± 0.13</td>
</tr>
<tr>
<td>Acidic steroids (mg/3d)</td>
<td>28.6 ± 2.6</td>
<td>29.8 ± 2.0</td>
</tr>
<tr>
<td>Neutral steroids (mg/3d)</td>
<td>71.9 ± 8.1</td>
<td>125 ± 11</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>65.2 ± 7.1</td>
<td>72.2 ± 7.1</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>6.64 ± 1.52</td>
<td>53.3 ± 3.9</td>
</tr>
<tr>
<td>Total steroids* (mg/3d)</td>
<td>100 ± 9</td>
<td>155 ± 9</td>
</tr>
</tbody>
</table>

* Each value is the mean ± SEM of six rats per group.

### Table II. Effects of a Casein Tryptic Hydrolyzate (CTH) and Soyprotein Peptic Hydrolyzate (SPH) on Cholesterol Uptake in CaCo-2 Cells*

<table>
<thead>
<tr>
<th>Cholesterol uptake in CaCo-2 cells (pmol/dish/20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTH (1 mg/ml)</td>
</tr>
<tr>
<td>(10 mg/ml)</td>
</tr>
<tr>
<td>SPH (1 mg/ml)</td>
</tr>
<tr>
<td>(10 mg/ml)</td>
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</tbody>
</table>

* Each value is the mean ± SEM of 3 determinations.

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1. Saeki et al. 21.  
2. Statistical significance (Student's t-test) compared with the CTH group is indicated as follows: *** p < 0.01; ** p < 0.001.  
3. HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein. Values were calculated as follows: LDL + VLDL-cholesterol = total cholesterol - HDL-cholesterol.  
4. Total steroids = acidic steroids + neutral steroids.
tinal epithelial cells. However, there have so far been few experimental studies to evaluate any effects of peptides on cholesterol absorption by using cultured intestinal cells. We found for the first time that SPH directly inhibited the absorption of micellar cholesterol by using the CaCo-2 cultured cell strain in the present study. We thus propose a new model for evaluating the changes in cholesterol absorption induced by dietary components.

Cholesterol is rendered soluble in bile salt mixed micelles and then absorbed.29) Iwami et al.30) have shown a correlation between the hydrophobicity of the protein hydrolyzate and its binding capacity to bile acid, and pointed out the possibility that a peptide with binding capacity to bile acid could inhibit the reabsorption of bile acid in the small intestine and decrease the blood cholesterol level. However, our present results clearly demonstrate that the hypocholesterolemic action of SPH was induced by inhibiting the cholesterol absorption by direct interaction between cholesterol mixed micelles and peptides or protein. Studies are now in progress to obtain further information on the mechanism for the SPH-induced inhibition of cholesterol absorption in CaCo-2 cells. Our experimental system to evaluate cholesterol absorption with CaCo-2 cells is very useful for clarifying the molecular mechanism for the inhibitory effect of soyprotein or SPH on cholesterol absorption from the small intestine, which has previously been unknown, and can also be expected to greatly facilitate an elucidation of the effects of various food constituents on cholesterol absorption in future.

References