Effects of Dietary Fish Protein, Soybean Protein and Casein on Cholesterol Turnover in Rats

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Summary The effects of dietary fish, soybean protein and casein on cholesterol turnover were compared in rats. After the injection of [14C]cholesterol into the rats, the specific activities of radioactive cholesterol in feces were followed for 4 weeks. The cholesterol half-lives calculated from the decay curves of the specific activities were 14.7 and 14.6 days in rats fed fish protein and soybean protein, respectively. These were shorter than the half-life (17.4 days) in casein-fed controls. The fish and the soybean protein feedings significantly increased the fecal excretions of cholesterol and coprostanol, respectively, and lowered the plasma cholesterol level, as compared with casein feeding. In addition, both fish and soybean protein feedings also increased the excretion of bile acids. The stimulation of cholesterol metabolism and the increased excretions of cholesterol and its metabolites by feeding fish or soybean protein appear to play important roles in the hypocholesterolemic effects.

Key Words cholesterol metabolism, fish protein, soybean protein, casein

The effects of dietary protein on hypercholesterolemia have been reported by many groups (1–17). Animal protein is generally considered to be more cholesterolemic than vegetable protein. Rats fed soybean protein showed lower serum and liver cholesterol levels as compared with the levels for the casein diet. One of the reasons for this may be that the less efficiently absorbable coprostanol was formed to a great extent in rats fed soybean protein than in those fed casein (13, 14). It was also suggested that the difference in the amino acid composition between animal and vegetable protein may be responsible for the different response of serum chole-
Although the hypocholesterolemic effects of soybean protein have been compared with those of casein, the effects of other animal proteins on cholesterol metabolism have not been investigated sufficiently. In the present study, the effects of dietary proteins including fish protein on cholesterol turnover in the whole body of rat were compared.

MATERIALS AND METHODS

Male Wistar rats, 5 weeks old (Awazu Animals, Osaka), were fed on three kinds of diet containing casein, soybean protein isolate (Fujipro R, Fuji Oil Co., Osaka; 90% protein, 4% salts, 3.5% carbohydrate, 0.3% fat and minor constituents) and fish protein isolate (from pollack, Theragra chalcogramma (Pollas); 86.5% protein, 4% salts, 0.1% fat and minor constituents) as protein sources. The diet consisted of 63.9% sucrose, 18% protein isolate, 5% olive oil, 9% cellulose, 4% salt mixture, 0.1% choline chloride and vitamins. Rats were injected intraperitoneally with 2 μCi [4-14C]cholesterol (50–60 mCi/mmol, New England) dissolved in 1 ml of 0.3% Tween 80 per 100 g body weight 2 weeks after being separated into three groups. The animals were maintained on an automatic lighting schedule from 7 a.m. to 7 p.m. at 24°C. The fecal excretions of neutral sterols and bile acids were traced.

Feces collected for 1- to 7-day periods (feces collected at days 1, 2, 4, 7, 10, 21 and 28) after the injection were dried in an oven at 105 to 110°C for 3 h and ground into powder. The feces were extracted in a Soxleht apparatus with acetone–absolute ethanol (1:1) for 48 h. The samples were further extracted with 0.1 N HCl in ethanol for 24 h. An aliquot of the combined extracts was evaporated to dryness, and the neutral sterols and bile acids were quantified by gas chromatography. Sterols were trimethylsilylated and injected into a Hitachi K-53 gas chromatograph equipped with a hydrogen flame detector. A column of SE-30, 1% on chromosorb W (80–100 mesh) packed in a 2 m glass tube, was maintained at 245°C with a flow of nitrogen. Bile acids were extracted from feces and analyzed by gas chromatography according to Uchida et al. A column of QF-1, 1% on chromosorb W (80–100 mesh) packed in a 2 m tube, was maintained at 230°C with a flow of nitrogen.

For the determination of specific activities of cholesterol (cpm/mg) in feces, cholesterol was collected by gas chromatograph without the hydrogen flame detector. The aliquots of cholesterol were assayed for radioactivity and mass by gas chromatography. The specific activities of cholesterol were plotted against time on semilogarithmic graph paper. The half-life of cholesterol was determined from the curve, essentially according to the method of Lindstedt.

Plasma and liver lipids were extracted according to Folch and their cholesterol contents were determined using the color reagent of Zak. The triglyceride contents were determined according to Fletcher and the phospholipids, by phosphorus determination.

For assay of trypsin inhibitor activity in soybean protein, the hydrolytic
activity of trypsin for N-a-benzoyl-DL-arginine-p-nitroaniline hydrochloride was measured in the absence and the presence of various amounts of soybean protein, essentially according to Erlanger (25). The inhibitory activity was estimated from the balance of the trypsin activities. The nitrogen of protein and feces was measured by the micro Kjeldahl method (26).

The data were analyzed by the Student's t-test (27).

RESULTS AND DISCUSSION

The rats were fed on dietary protein regimens for 2 weeks before the injection of [14C]cholesterol and for another 4 weeks after the injection. When the animals were injected with radioactive cholesterol after being fed the experimental diets for 2 weeks, body weight was lower in the soybean group than in the casein and the fish protein groups (Table 1). Soybean protein appears to decrease weight gain in young animals (from 5 to 7 weeks old). However, the weight gains for the 4 weeks (experimental period) after reaching 7 weeks of age, were not significantly different among the groups. The weights of feces relative to body weights tended to increase in the soybean group.

The fecal excretion of nitrogen was greater in the soybean group than in the

| Table 1. Effects of dietary proteins on body weight, liver weight and fecal excretion of nitrogen. |
|------------------------------------------------------|
| Dietary protein | Casein | Soybean | Fish |
|------------------------------------------------------|
| Body weight (g) | 174 ± 4.50 | 144 ± 10.4b | 174 ± 4.78e |
| 2 weeks1 | 306 ± 2.00 | 260 ± 7.26d | 307 ± 8.01f |
| 6 weeks1 | 132 ± 6.50 | 116 ± 11.3 | 133 ± 6.16 |
| Weight gain (g/4 weeks) | 15.8 ± 0.28 | 12.1 ± 0.86c | 16.4 ± 0.29f |
| Liver weight (g) | 1.45 ± 0.45 | 1.45 ± 0.43 | 1.35 ± 0.48 |
| Fecal dry weight (g/day)2 | 5.10 ± 0.30 | 7.75 ± 0.07d | 3.67 ± 0.13eg |
| Fecal excretion of nitrogen (% for intake) | 7.77 ± 0.28 | 9.19 ± 0.77a | 6.06 ± 0.61bf |

Mean ± SD (n = 3). As the results were reproduced approximately twice, the results for the animals shown in Fig. 1 are shown in the table. Male Wistar rats, 5 weeks old, were separated into three dietary groups: casein, soybean protein and fish protein. Two weeks after the separation, cholesterol turnover was measured for the following 4 weeks (2-6 weeks after the separation). The animals were then killed and liver weights were measured. 1Weeks after the separation of dietary groups. 2The mean values of fecal dry weights during the 4-week period. Significantly different from casein, a p < 0.05, b p < 0.02, c p < 0.01, d p < 0.001. Significantly different from soybean, e p < 0.02, f p < 0.01, g p < 0.001.

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Fig. 1. Semilogarithmic plot of cholesterol specific activity in rat feces after the injection of $[^{14}C]$cholesterol. Each individual rat was injected intraperitoneally with 2 $\mu$Ci $[^{14}C]$cholesterol/100 g body weight and the fecal excretion of cholesterol in terms of specific activity (cpm/mg) was traced. The regression lines were calculated by the method of least squares. The cholesterol half-lives were calculated from these lines and shown in the text. The triangles, black circles and white circles show the results for casein, fish protein and soybean protein groups, respectively.

other groups, as shown in Table 1. Fish protein appears to be more digestive in rats than casein. In the soybean protein used, a small activity of trypsin inhibitor was found. The inhibitory activity of 1 g soybean protein corresponded to that of 1.08 mg of crystalline trypsin.

**Turnover of injected radioactive cholesterol**

The specific activities of $[^{14}C]$cholesterol in the feces of the three dietary protein groups were measured and plotted against time on semilogarithmic graph paper. During the early stage after the injection of isotopic cholesterol, the isotopic cholesterol appears to be incompletely equilibrated with the endogenous cholesterol. Therefore, the decay curves from 7 to 28 days after the injection were calculated by the least square method (Fig. 1). The half-lives were 17.4, 14.6 and 14.7 days for the casein, soybean and fish protein groups, respectively. Dietary soybean and fish proteins appear to reduce the half-life of cholesterol as compared with casein.

**Fecal excretion of cholesterol and its metabolites**

The fecal excretions of cholesterol and its metabolites are shown in Table 2. The fecal excretion of cholesterol was greater in the soybean group, and particularly in the fish group, than in the casein group. The excretion of coprostanol was greater in the soybean group than in the other groups. Further, bile acid excretion was increased by increasing deoxycholic acid and hyodeoxycholic acid intake in the soy protein group. Consequently, the excretion of total steroids increased in the following order: casein, fish and soy. Further, Table 3 shows the cumulative

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Table 2. Effects of dietary proteins on fecal sterol and bile acid excretion.

| Sterols          | Casein       | Soybean      | Fish         |
|------------------|--------------|--------------|--------------|
|                  | (mg/day/rat) |              |              |
| Cholesterol      | 0.69 ± 0.17  | 1.09 ± 0.23  | 1.45 ± 0.34a |
| Total sterols    | 1.34         | 2.89         | 2.48         |
| Bile acids       |              |              |              |
| Lithocholic acid | 0.31 ± 0.08  | 0.36 ± 0.05  | 0.29 ± 0.04  |
| Deoxycholic acid | 0.40 ± 0.08  | 0.68 ± 0.08b | 0.46 ± 0.10d |
| Chenodeoxycholic acid | 0.10 ± 0.02 | 0.16 ± 0.04  | 0.18 ± 0.04a |
| Hyodeoxycholic acid | 0.58 ± 0.08 | 0.80 ± 0.10a | 0.92 ± 0.18a |
| Cholic acid      | 0.32 ± 0.01  | 0.38 ± 0.04  | 0.16 ± 0.04c |
| Others           | 0.15 ± 0.03  | 0.18 ± 0.04  | 0.18 ± 0.04  |
| Total bile acids | 1.86         | 2.56         | 2.19         |
| Sterols and bile acids | 3.20   | 5.45         | 4.67         |

Mean ± SD (n = 3). The data show fecal amounts of sterols and bile acids per day per rat during the 4–6 week period following the separation of dietary groups. The experiments were repeated twice. One set of results is shown in the table, as the results were the same. Significantly different from casein, *p < 0.05, b p < 0.02, c p < 0.01. Significantly different from soybean, d p < 0.05, e p < 0.01.

Table 3. Effects of dietary proteins on excretions of injected [14C]cholesterol into fecal sterols and bile acids.

| Dietary protein | Casein       | Soybean      | Fish         |
|-----------------|--------------|--------------|--------------|
|                 | (cpm × 10⁻⁵/28 days/rat) |              |              |
| Neutral sterols | 4.37 ± 3.44  | 11.4 ± 0.82a | 10.1 ± 0.13a |
| Bile acids      | 6.36 ± 0.50  | 9.45 ± 0.38b | 10.1 ± 0.13c |

Mean ± SD (n = 5). The data show the cumulative excretions of radioactivities into fecal sterol and bile acid fractions for 28 days after the injection of [4-¹⁴C]cholesterol. Significantly different from casein, *p < 0.05, b p < 0.01, c p < 0.001.

Excretions of radioactivities into fecal sterols and bile acids during the experimental period (28 days) after the injection of [¹⁴C]cholesterol into rats. The excretions of radioactivities into fecal sterols and also bile acids were greatly enhanced in both the soy and fish protein groups in comparison with the casein group. Thus, the excretions of radioactivity confirmed the increases in fecal excretions of cholesterol and its metabolites in the former two groups. Soybean protein feeding appears to
Table 4. Effects of dietary proteins on lipid levels in plasma and liver.

|                     | Casein      | Soybean     | Fish        |
|---------------------|-------------|-------------|-------------|
| **Plasma**          |             |             |             |
| Cholesterol (mg/ml) | 1.30 ± 0.19 | 0.81 ± 0.22a | 0.91 ± 0.03a |
| Triglycerides (mg/ml)| 2.02 ± 0.52 | 1.88 ± 0.40  | 1.52 ± 0.16  |
| Phospholipids (µmol/ml) | 2.81 ± 0.05 | 2.84 ± 0.38  | 2.96 ± 0.08  |
| **Liver**           |             |             |             |
| Cholesterol (mg/g)  | 4.59 ± 0.40 | 3.70 ± 0.31a | 4.06 ± 0.18  |
| Triglycerides (mg/g) | 59.1 ± 1.15 | 18.1 ± 5.99c | 68.7 ± 2.91bd |
| Phospholipids (µmol/mg) | 57.2 ± 4.45 | 55.5 ± 4.80  | 55.8 ± 1.56  |

Mean ± SD (n=3). The experiments were repeated five times with very similar results. The data for the animals shown in Tables 1 and 2 and in Fig. 3 are given. Significantly different from casein, a p<0.05. b p<0.01, c p<0.001. Significantly different from soybean, d p<0.001.

stimulate cholesterol excretion due to an increase in the formation of coprostanol, less efficiently absorbable, by intestinal microflora. The formation of hyodeoxycholic acid from cholic acid by microflora was also increased. The stimulation of coprostanol formation due to undigested soybean protein has been reported by other groups (5, 28). The soybean protein used in the present study contained very little saponin (less than 4 g/kg). Small amounts of saponin do not seem to produce a significant effect on plasma cholesterol levels (29). On the other hand, we found trypsin inhibitor activity in the soybean protein. The trypsin inhibitor, which is not digestible (30), may disturb soybean protein digestion. Rats fed soybean protein excreted rather large amounts of feces in consideration of body weight, as shown in Table 1. As the fecal excretion of cholesterol was greater in the fish group than in the others, the absorption of cholesterol in the enterohepatic circulation appears to be disturbed by the feeding of fish protein. The reason for this is not known at the present stage. Undigested soybean protein may stimulate the excretion of cholesterol and metabolites.

At the end of the experiment (6 weeks after feeding the protein diet), the lipid levels of plasma and liver were measured. As shown in Table 4, plasma cholesterol levels were lower in rats fed soybean or fish protein than in those fed casein. Liver triglycerides were markedly reduced by feeding soybean protein, but not by feeding fish protein.

Sugano et al. (5) found that although the increase of coprostanol formation was observed in rats given soybean protein, the increase was not observed in rats given the amino acid mixture simulating soybean protein, whereas plasma HDL-and VLDL-cholesterol decreased in the rats. They suggested that the differences in

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the amino acid composition between animal and vegetable protein may be responsible for the different response of plasma cholesterol. Several workers have attempted to attribute the hypocholesterolemic effect of vegetable proteins to specific amino acids but with conflicting results (5, 10–12). Soybean protein appears to have an effect on cholesterol excretion besides the effects of the corresponding amino acid mixture on plasma cholesterol (9). In the present experiment we found that plasma cholesterol in rats respectively fed fish protein and soybean protein was also lower than in those fed casein.

We found that the overall cholesterol turnover (including metabolism and excretion of cholesterol) was lower in the whole bodies of rats fed soybean or fish protein than in those fed casein. Sugano et al. (9) and Carroll et al. (8) reported that soy protein stimulated the turnover of cholesterol in comparison with casein. The increased excretions of sterols and bile acids were the significant effects common to fish and soy protein feedings. Therefore, in the present stage, it is suggested that the hypocholesterolemic effects of fish may be ascribed to the stimulation of the metabolism of cholesterol to bile acids, and to the increased excretion of cholesterol and its metabolites.

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