Effects of Casein and Soy Protein on Accumulation of Cholesterol and Dolichol in Rat Liver (43575)

MASATAKA ISHINAGA,[1] MIWAKO HAMADA, KUMI OHNAKA, KAZUKO FUKUNAGA, AND YOSHIKO MINATO
Department of Food and Nutrition, Hiroshima Women's University, 1-1-71 Ujina-Higashi, Minami-ku, Hiroshima 734, Japan

Abstract. **A diet containing 15% (w/w) fat and 20% (w/w) of either casein (CAS) or soy protein (SOY) was fed to 4-week-old rats for a period of 18 months. The effects of these dietary proteins on the accumulation of cholesterol and dolichol in livers were studied. After 1 month, the amount of liver cholesterol was about 5 mg/g of liver. After an additional 5 months of feeding, there was a slight decrease in cholesterol per gram of liver (3.6 mg/g of liver in CAS-fed rats and 2.6 mg/g of liver in SOY-fed rats). However, after 18 months, there were a remarkable increase (7.5 mg/g of liver) in CAS-fed rats and only a slight increase in SOY-fed rats. The proportions of liver cholesterol ester in rats fed the CAS diet were 60–70% of the total cholesterol during the experimental period, but in the case of the SOY diet, only rats fed the diet for 1 month showed a high level, 70%, of cholesterol ester.**

The amounts of liver dolichol in rats fed the CAS and SOY diets after feeding for 18 months were 60 μg and 47 μg of liver, respectively. There was a 1.5-fold increase in both diets for a period of 18 months. The proportions of liver dolichyl fatty ester in rats fed the CAS diet were 35–40% of the total dolichol during the experimental period, but in the case of the SOY diet, only rats fed the diet for 1 month showed a high level, 36%, of dolichyl fatty ester. The proportions of dolichol ester in rats fed the SOY diet were 25–30% after 6 and 18 months of feeding.

These observations indicated that the SOY diet depresses the accumulation of both liver dolichol and cholesterol. [P.S.E.B.M. 1993, Vol 203]

Dolichols are α-saturated long chain polyisoprenoid alcohols present on all eukaryotic organisms (1). Accumulation of free dolichols and dolichyl fatty esters has been observed in many animal tissues with aging (2–4). Dolichyl phosphate, however, does not do so in rats (2, 3). The physiologic significance of accumulation of free dolichols and dolichyl fatty esters is not yet known. However, remarkable accumulation of dolichols was observed in Alzheimer's disease and also certain other neurologic diseases in humans (5, 6). Hence, it is assumed that accumulation of excess amounts of dolichols in cells might impair certain important cellular functions.

Although hepatic hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase is the regulatory enzyme for sterol biosynthesis, it also provides intermediates for the biosynthesis of dolichol and ubiquinone (7). In mice fed a diet containing cholesterol, HMG-CoA reductase in the liver is fully inhibited, whereas the synthesis of dolichol is decreased only 50% (8). In contrast, an increase in liver dolichol was observed in rats fed cholesterol (9). Hence, it appears that the effect of exogenous cholesterol on the biosynthesis of dolichol differs among animal species.

Sugano's group (10–12) reported that in spite of an increase in HMG-CoA reductase in rat liver, feeding soy diet reduces the amounts of liver and plasma cholesterol. In this paper, we describe the changes in the accumulation of liver cholesterol and dolichol after feeding casein (CAS) and soy protein (SOY) diets without adding exogenous cholesterol to the diets for a period of 18 months.

Materials and Methods

Animals and Diets. Four-week-old male Wistar rats were used. The rats were divided into two groups

[1] To whom requests for reprints should be addressed at Department of Food and Nutrition, Hiroshima Women's University, 1-1-71 Ujina-Higashi Hiroshima, 734 Japan.

Received September 17, 1992. [P.S.E.B.M. 1993, Vol 203]
Accepted December 23, 1992.

0037-9727/93/2031-0074$3.00/0

of five or six each. They were fed *ad libitum* two experimental diets containing CAS or SOY for 1, 6, and 18 months. The diets contained, by weight (in %): dietary protein (either SOY or CAS), 20.0; lard, 13.5 (containing 0.1% butylhydroxyltoluene); corn oil, 1.5; sucrose, 52; mineral mixture, 4[2]; vitamin mixture, 2[3]; and cellulose powder, 2. The soy protein isolate (Fuji Pro) was kindly provided by Fuji Oil (Osaka, Japan). Casein, the mineral mixture (Harper), and the vitamin mixture (Harper) were obtained from Oriental Yeast Co. (Tokyo, Japan). The diets were prepared every 3 days and stored in the dark at 5°C. The rats were provided with fresh food every day.

Materials. Dolichol from porcine liver and polyprenol 20 were purchased from Sigma (St. Louis, MO). Polyprenol 23 was purchased from Larodan Fine Chemicals AB (MalmÖ, Sweden). The amount of polyprenol 23 was determined as described previously (13). A Cosmosil column (4.8 mm × 15 cm), cholesterol, and 5α-cholestane were purchased from Nakarai Tesque (Kyoto, Japan). Sodium methoxide, hexamethyl disilazane, and trimethylchlorosilane were products of Pierce (Rockford, IL). Standard fatty acid methyl esters were products of Nu Check Prep, Inc. (Elysian, MN). The guard column (Lichrosphere C18) was purchased from E. Merck (Darmstadt, Germany). Sep-Pak silica was obtained from Waters Associates (Milford, MA). Methanol and isopropanol were of high-performance liquid chromatography (HPLC) grade. All other chemicals were of analytical grade.

Extraction and Isolation of Lipids. Rats were sacrificed at each time point by decapitation and their livers were removed immediately and homogenized in a cold chloroform/methanol (1:2) solution containing 0.01% butylhydroxyltoluene in a biomixer (Nihon Seiko Co., Tokyo, Japan). Lipids were extracted as described by Arthur and Sheltawy (modification of the method of Bligh and Dyer; 14). The extraction procedure was carried out three times. The crude lipids were stored at −80°C until used. The crude lipids (corresponding to 0.3 g of liver) were passed through Sep-Pak silica, and then the neutral lipid fraction containing cholesterol, cholesteryl ester, free dolichols, and dolichyl fatty esters was eluted with 30 ml of chloroform. These lipids were separated by HPLC. HPLC analysis was carried out according to the method described previously (13). Polyprenol 23 and 5α-cholestane were added to these fractions as internal standards. The amounts of dolichol were calculated from polyprenol 23 as described previously (13). The proportions of choresteryl ester in the total cholesterol and dolichyl fatty ester in the neutral dolichol have been estimated. The fatty acid composition of dolichyl fatty esters was also estimated.

Saponification and Trimethylsilylation. A portion of crude lipids containing 5α-cholestane and polyprenol 23 as internal standards and each lipid fraction obtained by HPLC were saponified as described in a previous paper (13). After saponification, a part of each was analyzed for dolichol by HPLC and the other part was trimethylsilylated by the method of Christie (15) for analysis of cholesterol.

Fatty Acid and Cholesterol Analysis. The dolichyl fatty ester fraction obtained on HPLC was methylated as described in the previous paper (13). Methyl esters were determined by gas liquid chromatography (Shimadzu GC-9A) on a G-300 wide-bore capillary column (Chemicals Inspection and Testing Institute, Tokyo, Japan) with an He flow rate of 20 ml/min and the temperature was programmed from 160°C to 200°C. Trimethylsilylated cholesterol was also determined by gas liquid chromatography on a column packed with 5% OV-17 (3.5 mm × 2 m) with an N_2 flow rate of 35 ml/min at 290°C using 5α-cholestane as a calibration standard.

The values are means ± SE for five or six rats. The statistical significance of the differences was determined by Student's *t* test.

Results

Body and Liver Weights. After feeding for 18 months, the body weights of rats fed CAS and SOY diets were 1012 ± 17 g and 890 ± 35 g, respectively. Although there was no significant difference in the body weight between the CAS and SOY diets, the body weights of rats fed the CAS diet tended to be higher than those of rats fed the SOY diet. After feeding for 18 months, the liver weights of rats fed the CAS and SOY diets were 29.0 ± 1.4 g and 20.7 ± 0.8 g, respectively. The difference was significant ($P < 0.01$).

Cholesterol. Initially, cholesterol in the livers of rats fed the CAS diet increased transiently (4.8 mg/g of liver) at the end of the first month and then decreased up to 3.6 mg/g of liver at the end of sixth month. Then, a remarkable increase (7.5 mg/g of liver) was observed at the end of 18th month (Fig. 1A). On the other hand, a similar transient increase in cholesterol per gram of liver was observed at the end of first month in rats fed the SOY diet. At the end of the sixth month, the amount of cholesterol per gram of liver was significantly lower ($P < 0.01$) in rats fed SOY diet than in the CAS-fed rats. After feeding SOY diet for an additional 12 months, there was only a slight increase in cholesterol,

[2] The mineral mixture contained (in g/kg diet): $CaHPO_4 \cdot 2H_2O$, 0.17; KH_2PO_4, 13.72; NaCl, 10.02; Fe-citrate, 0.25; $MgSO_4 \cdot 7H_2O$, 3.99; $ZnCl_2$, 0.008; $MnSO_4 \cdot 4H_2O$, 0.048; $CuSO_4 \cdot 5H_2O$, 0.062; KI, 0.0002; $CaCO_3$, 11.72; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.001.

[3] The vitamin mixture contained (in mg/kg diet): vitamin A acetate, 9320 IU; vitamin D, 4660 IU; vitamin E acetate, 240; vitamin K, 1.2; vitamin $B_1 \cdot HCl$, 11.8; vitamin B_2, 11.8; vitamin $B_6 \cdot HCl$, 5.8; vitamin B_{12}, 0.04; vitamin C, 117.6; biotin, 0.2; folic acid, 0.4; pantothenic acid-Ca, 47; niacin, 58.8; *myo*-inositol, 235.2.

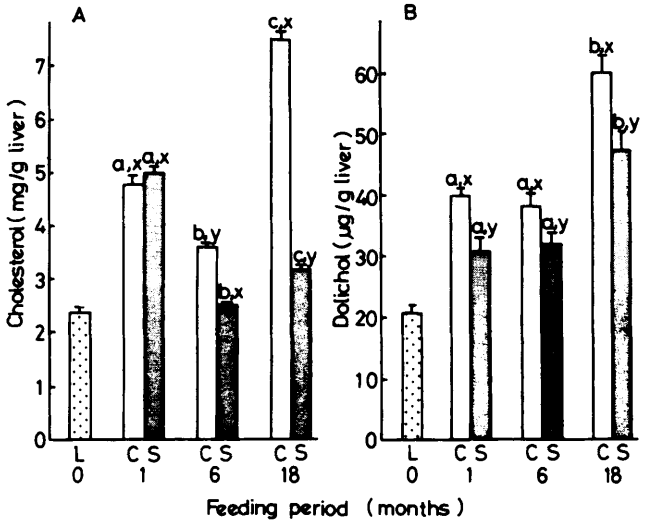

Figure 1. Variation with the feeding period and diet in the contents of (A) total cholesterol and (B) total dolichol in rat liver. L, lab chow; C, casein diet, S, soy protein diet. Means for the same diet with different superscripts (a, b, c) differ significantly. Means for the same feeding period with different superscripts (x, y, z) differ significantly ($P < 0.05$).

Table I. Percentages of Esterified Cholesterol and Dolichol of Total Cholesterol and Dolichol in Rat Liver (w/w)[a]

Feeding period (mo)	Cholesteryl ester		Dolichyl ester	
	Casein (%)	Soy protein (%)	Casein (%)	Soy protein (%)
0	16.1 ± 0.9	16.1 ± 0.9	22.1 ± 1.9	22.1 ± 1.9
1	61.2 ± 2.1*	70.0 ± 2.0*	39.5 ± 2.0*	36.6 ± 1.0*
6	66.9 ± 1.8*	38.0 ± 1.5†	34.6 ± 1.5*	30.0 ± 1.0†
18	73.0 ± 1.8†	23.7 ± 1.1‡	38.1 ± 1.1*	24.6 ± 0.3‡

[a] Amounts of total cholesterol and dolichol were presented in Figure 1. Values are means ± SE for five or six rats. Means in the same column with different symbols (*, †, ‡) differ significantly ($P < 0.05$).

from 2.7 to 3.2 mg/gram of liver.

Table I shows the proportion of cholesteryl ester in livers of rats. Sixty to seventy percent of the total cholesterol was cholesteryl ester in rats fed the CAS diet. In the case of the SOY diet, only when the total cholesterol was higher at the end of first month was the cholesteryl ester level also higher than 50%.

Dolichol. Neutral dolichol (free dolichol + dolichyl fatty ester) in liver of rats fed the CAS diet increased from 22 to 43 μg/g of livers at the end of first month (Fig. 1B). The amount of dolichol increased to 60 μg/g of liver (1.5-fold) at the end of 18th month. On the other hand, the level of neutral dolichols in livers of rats fed the SOY diet for 1 month was 31 μg/gram of liver. This value was significantly lower than that in the case of the CAS diet. The amount of dolichol had apparently not changed after 6 months of feeding. The amount of dolichol increased to 47 μg/g of liver after feeding 18 months and was 80% in the rats fed the CAS diet.

Table I shows the proportions of dolichyl fatty esters of total dolichol in rat liver. The percentage of dolichyl fatty esters was less than 30% in rats fed the SOY diet for 6 and 18 months. Dolichyl fatty esters, up to 35–40%, were observed in rats fed the CAS and the SOY diets for a 1-month period.

Distribution of the dolichol isoprenologs shifted toward those of lower chain length during aging and the composition was not different among diets (data not shown).

The fatty acid composition of dolichyl fatty esters in both the CAS- and SOY-fed rats was not very different and was apparently affected by lard in the diets (Table II). Approximately 50% of oleic acid and 12% of linoleic acid were observed in both diets. The fatty acid composition did not change during the experimental period (data not shown).

Discussion

It has been well established that a common regulatory step of the biosynthesis of cholesterol and dolichol is at the level of the HMG-CoA reductase (7, 8, 16). Rats and mice fed a diet containing cholesterol exhibited contradictory results regarding the accumulation of liver dolichol (8, 9). Since dietary cholesterol suppresses the activity of HMG-CoA reductase (8), we used a CAS or SOY diet without added cholesterol. As shown in Figure 1, the amounts of neutral dolichol were higher in CAS-fed rats than in SOY-fed rats during all feeding periods. The amounts of total cholesterol were also higher in CAS-fed rats, except at 1 month. The transient increases of these lipids in both diets at the 1-month period may be due to rapid synthesis of lipids in 4-week-old rats, because diets containing 15% fat and 52% sucrose were fed. Our finding is that the amount of dolichol increases with the amount of cholesterol in CAS-fed rats, which is in agreement with the report of Tavares *et al.* (9). We think that only CAS diet, but not SOY diet, may enhance the biosynthesis of both dolichol and cholesterol in the liver. Further-

Table II. Fatty Acid Composition of Dolichyl Fatty Esters in Liver of Rats Fed for 6 Months (w/w)[a]

	0 Month (%)	Casein (%)	Soy protein (%)
16:0	27.5 ± 0.3	26.9 ± 0.8	28.7 ± 0.6
16:1	5.2 ± 0.2	4.5 ± 0.2	3.7 ± 0.3
18:0	3.5 ± 0.0	4.0 ± 0.2	6.9 ± 0.2
18:1	29.6 ± 0.7	50.8 ± 1.4	44.6 ± 1.0
18:2	27.5 ± 0.6	11.9 ± 0.2	12.1 ± 0.5
18:3	1.0 ± 0.2	0.3 ± 0.0	0.7 ± 0.1
20:4	1.4 ± 0.1	1.0 ± 0.1	2.3 ± 0.2
22:5	1.0 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
22:6	3.3 ± 0.3	0.5 ± 0.1	0.8 ± 0.2

[a] Values are means ± SE for five or six rats.

more, excretions of dolichol and cholesterol from the liver might be slower in CAS-fed rats than in SOY-fed rats. Choi *et al.* (12) indicated enhanced excretion of cholesterol from the liver of SOY-fed rats compared with CAS-fed rats. We believe that dolichol may be excreted with cholesterol as lipoprotein from the liver. Since the diet- and age-dependent differences in the amounts of liver dolichol were smaller than those of cholesterol, it was assumed that dolichol was excreted from the liver more slowly than cholesterol. This idea was supported by the fact that the half-life of hepatic dolichol is about 5 days (17).

Acylating enzymes of cholesterol and dolichol are located in the endoplasmic reticulum of the liver (18, 19). The activity of acyl-CoA:cholesterol acyltransferase increases 2-fold in the liver of cholesterol-fed rats. This could account for the increased amount of cholesteryl ester observed in those rats (20). Imaizumi *et al.* (21) also reported that the hepatic level of cholesteryl ester is 90% of the total cholesterol in cholesterol-fed rats. Although Kabakoff and Kandutsch (8) did not determine the ratio of free cholesterol to cholesteryl ester in cholesterol-fed mice, they observed the accumulation of dolichyl fatty esters in liver. Accumulation of cholesterol in hepatic cells probably induces an increase in the activity of acyl-CoA:cholesterol acyltransferase. In our experiment, the CAS diet might have enhanced the activity of acyl-CoA:cholesterol acyltransferase. As shown in Table I, both in CAS-fed and SOY-fed rats, the percentage of dolichyl fatty ester is higher when the percentage of cholesteryl ester is also higher. These results suggest that acylation of cholesterol coordinates acylation of dolichol.

Our results indicate that soy protein depresses the accumulation of liver dolichol together with cholesterol when compared with that of casein-fed rats.

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

1. Rip JW, Rupar CA, Ravi K, Carroll KK. Distribution, metabolism and function of dolichol and polyprenols. Prog Lipid Res **24**:269–309, 1985.
2. Keller RK, Nellis SW. Quantitation of dolichyl phosphate and dolichol in major organs of the rat as a function of age. Lipids **21**:353–355, 1986.
3. Kalen A, Appelkvist E-L, Dallner G. Age-related changes in the lipid compositions of rat and human tissues. Lipids **24**:579–584, 1989.
4. Daniels I, Hemming FW. Changes in murine tissue concentrations of dolichol and dolichol derivatives associated with age. Lipids **25**:586–593, 1990.
5. Wolfe LS, Ng Ying Kin NMK, Palo J, Haltia M. Raised levels of cerebral cortex dolichols in Alzheimer's disease. Lancet **ii**:99, 1982.
6. Ng Ying Kin NMK, Palo J, Haltia M, Wolfe LS. High levels of brain dolichols in neuronal ceroid-lipofuscinosis and senescence. J Neurochem **40**:1465–1473, 1983.
7. Brown MS, Goldstein JL. Multivalent feedback regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res **21**:505–517, 1980.
8. Kabakoff BD, Kandutsch AA. Depression of hepatic dolichol levels by cholesterol feeding. J Lipid Res **28**:305–310, 1987.
9. Tavares A, Coolbear T, Hemming FW. Increased hepatic dolichol and dolichyl phosphate-mediated glycosylation in rats fed cholesterol. Arch Biochem Biophys **207**:427–436, 1981.
10. Nagata Y, Ishiwaki N, Sugano M. Studies on the mechanism of antihypercholesterolemic action of soy protein and soy protein-type amino acid mixtures in relation to the casein counterparts in rats. J Nutr **112**:1614–1625, 1982.
11. Sugano M, Ishida T, Koba K. Protein-fat interaction on serum cholesterol level, fatty acid desaturation and eicosanoid production in rats. Lipids **118**:548–554, 1988.
12. Choi Y-S, Ikeda I, Sugano M. The combined effects of dietary proteins and fish oil on cholesterol metabolism in rats of different ages. Lipids **24**:506–510, 1989.
13. Ishinaga M, Yamauchi T, Egusa K, Kuroda K. Changes of dolichol and dolichyl fatty acyl esters during the germination and development of soybeans. Biochem Cell Biol **70**:466–469, 1992.
14. Arthur G, Sheltawy A. The presence of lysophosphatidylcholine in chromaffin granules. Biochem J **191**:523–532, 1980.
15. Christie WW. Gas Chromatography and Lipids. Ayr, Scotland: The Oily Press, p78, 1989.
16. James MJ, Kandutsch AA. Inter-relationships between dolichol and sterol synthesis in mammalian cell cultures. J Biol Chem **254**:8442–8446, 1979.
17. Edlund C, Brunk U, Chojnacki T, Dallner G. The half-lives of dolichol and dolichyl phosphate in rat liver. Biosci Rep **8**:139–146, 1988.
18. Brown MS, Dana SE, Goldstein JL. Cholesterol ester formation in cultured human fibroblasts. J Biol Chem **250**:4025–4027, 1975.
19. Tollbom O, Valtersson C, Chojnacki T, Dallner G. Esterification of dolichol in rat liver. J Biol Chem **263**:1347–1352, 1988.
20. Grogan WM, Bailey ML, Heuman DM, Vlahcevic ZR. Effects of Perturbations in hepatic free and esterified cholesterol pools on bile acid synthesis, cholesterol 7-hydroxylase, HMG-CoA reductase, acyl-CoA:cholesterol acyltransferase and cytosolic cholesteryl ester hydrolase. Lipids **26**:907–914, 1991.
21. Imaizumi K, Sekihara K, Sugano M. Hypocholesterolemic action of dietary phosphatidylethanolamine in rats sensitive to exogenous cholesterol. J Nutr Biochem **2**:251–254, 1991.