Consumption of Casein Instead of Soybean Protein Produces a Transient Rise in the Concentration of Sphingomyelin in VLDL in Rats

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ABSTRACT In rats fed cholesterol-rich diets, dietary casein vs. soybean protein raises VLDL cholesterol concentrations. Because sphingomyelin may be an essential component of VLDL, we tested whether casein feeding would raise VLDL-sphingomyelin. Rats were fed cholesterol-rich semipurified diets containing either soybean protein (35 g/100 g) or casein for up to 21 d. Consistent with previous work, casein consumption increased hepatic and VLDL cholesterol concentrations. Dietary casein also significantly raised the amount of sphingomyelin in the VLDL fraction, but this effect was transient. Casein feeding transiently lowered LDL- and HDL-2-sphingomyelin concentrations. We suggest that an increase in hepatic VLDL secretion after casein consumption imposed an increased demand for sphingomyelin in the liver. The activity of key enzymes of sphingomyelin synthesis, i.e., serine palmitoyltransferase, phosphatidylcholine:ceramide phosphocholinetransferase and phosphatidylethanolamine:ceramide phosphoethanolaminetransferase and sphingomyelin degradation, i.e., acid sphingomyelinase, were enhanced and depressed, respectively, by casein consumption. Again these effects were transient. Thus, these data indicate that the extra sphingomyelin needed after short-term casein feeding came about through enhanced rates of biosynthesis and reduced rates of degradation in the liver. In addition, plasma transfer of sphingomyelin from HDL-2 to VLDL might have contributed to the increase in VLDL sphingomyelin in the casein-fed rats. This study shows that dietary casein vs. soybean protein transiently influences sphingomyelin metabolism in rats. J. Nutr. 129: 2119–2122, 1999.

KEY WORDS: sphingomyelin • dietary casein • liver • lipoproteins • rats

The feeding of semipurified diets containing casein instead of soybean protein produces hypercholesterolemia and atherosclerosis in rabbits (Carroll 1982, Kritchevsky 1979). Dietary casein enhances cholesterol absorption and reduces fecal bile acid secretion, leading to increased hepatic cholesterol levels. This elicits various reactions, including an increased output of VLDL by the liver, which results in an elevated concentration of VLDL cholesterol (Beynen 1990). In previous studies with rats fed diets containing extra cholesterol (Geelen et al. 1995), pectin (Bladergroen et al. 1999) or olive oil (Geelen and Beynen 1999), we showed that diet-induced changes in VLDL cholesterol levels were associated with parallel changes in the amount of sphingomyelin in the VLDL fraction. In this study, we tested whether dietary casein vs. soybean protein would increase VLDL-sphingomyelin in rats. We have attempted to describe how dietary casein affects hepatic sphingomyelin metabolism.

MATERIALS AND METHODS

Chemicals. C12-NBD ceramide was purchased from Molecular Probes (Eugene, OR). The origin of other chemicals has been described previously (Geelen et al. 1995).

Animals and diets. Female outbred Wistar rats (HsdCpb:Wu, Harlan-CPB, Zeist, The Netherlands), aged 3 wk, were used. They were housed in groups of three per cage in a room with a 12-h light:dark cycle (lights on, 0700–1900 h). All rats were fed a diet containing soybean protein for 7 d. The composition of the diet was as follows (g/100 g): soybean protein, 35; methionine, 0.3; coconut oil, 9; soybean oil, 1; cholesterol, 1; glucose, 44.8; cellulose, 3; calcium carbonate, 1.2; monosodium phosphate, 1.5; magnesium carbonate, 0.2; potassium chloride, 0.8; mineral premix, 1; and vitamin premix, 1.2. Before the ingredients were mixed, the cholesterol component of the diet was dissolved in the coconut part of the diet. The composition of the vitamin and mineral premixes was described by Verbeek et al. (1993). After 7 d (d 0 of the experiment), the rats were divided into two groups of 18 and one group of 6, stratified for body weight. One group of 18 rats continued to receive the diet with soybean protein; the other group of 18 rats was transferred to the same diet, but with 35.3 g casein/100 g replacing the soybean protein and methionine. Rats had free access to food and tap water.

Collection and preparation of samples. Samples were taken exactly as described before (Geelen et al. 1995). Lipoproteins were isolated from fresh plasma by density gradient centrifugation (Terpstra et al. 1981). VLDL, LDL and HDL were collected on the basis of their densities as before (Geelen et al. 1995). Isolated lipoprotein fractions were frozen and stored at −20°C until analysis.

Several pieces of liver were homogenized separately and used for lipid extraction and subcellular fractionation as described previously (Geelen et al. 1995).

Enzyme assays. Activities of sphingomyelinase (EC 3.1.4.12) were determined in liver preparations. Acid sphingomyelinase activ-
ity was determined in liver homogenates as described before (Geelen et al. 1995). Assays were conducted at pH 4.4 for 60 min at 37°C. Neutral sphingomyelinase activity was determined in isolated plasma membranes. The last-mentioned assay was performed similarly to the one for acid sphingomyelinase except that the buffer was 50 mmol/L Tris-HCl (pH 7.4) and the incubation was carried out in the presence of 40 mmol/L MgCl₂ for 20 min.

Determination of the activity of serine palmitoyltransferase (EC 2.3.1.50) was carried out as described (Geelen et al. 1995).

The activities of phosphatidylcholine:ceramide cholinephosphotransferase and phosphatidylethanolamine:ceramide ethanolamine-phosphotransferase were determined essentially as described by Vos et al. (1995). Briefly, the assay mixture contained the following in a total volume of 250 μL: 26 mmol/L 12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-lauryl-ceramide (NBD), 174 mmol/L egg phosphatidylcholine or phosphatidylethanolamine, 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 20 μmol Triton X-100/L and 50 μL purified plasma membrane. Control experiments demonstrated that the assays were linear with protein up to at least 200 μg of plasma membrane protein and with time, for at least 3 h. For routine purposes, assays were conducted for 60 min with ~100 μg of plasma membrane protein. Lipids were extracted according to Bligh and Dyer (1959) and separated by TLC on silica G-60 plates using chloroform/methanol/25% ammonia. The lipids were eluted from the plates. For quantification, the NBD-ceramides were excited at 465 nm and their fluorescence was measured at 530 nm. The fluorimetry was carried out with a Perkin Elmer Luminescence Spectrometer LS-2B.

**Chemical analyses.** Cholesterol and sphingomyelin in plasma, liver homogenates and lipoprotein fractions were isolated and quantified exactly as described previously (Geelen et al. 1995).

**Statistical analysis.** Results shown represent the means ± SD. The data within the two dietary groups for the three time points are independent so that statistical analysis of diet effects for each time point was performed by two-tailed Student’s t test. The level of significance was preset at P < 0.05.

**RESULTS**

Body weights of the rats fed soybean protein and casein did not differ significantly; on d 21 of the experiment, the values were 298 ± 35.5 and 313.9 ± 33.5 g, respectively. Liver weight was greater in the casein-fed group than in the soybean protein-fed group. On d 7 of the experiment, the relative liver weights in the groups given soybean protein and casein were 5.08 ± 0.31 and 5.50 ± 0.43 g/100 g body wt, respectively. On d 14 of the experiment, the values were 4.87 ± 0.27 and 5.54 ± 0.07 (P < 0.001) and on d 21, they were 4.86 ± 0.43 and 5.35 ± 0.32 g/100 g body wt (P < 0.05).

Plasma cholesterol was greater in the casein group than in the soybean group on d 21. The liver cholesterol level in casein-fed rats was significantly greater throughout the experiment (Fig. 1). Casein feeding did not significantly influence plasma and liver concentrations of sphingomyelin (Fig. 1).

As shown in Figure 2A, rats fed casein had more cholesterol in the VLDL fraction on d 14 and 21. Casein consumption induced a rapid increase in VLDL-sphingomyelin (Fig. 2B), but diminished LDL sphingomyelin (Fig. 2D). The sphingomyelin concentration of the HDL-2 particles was reduced by consumption of casein (Fig. 2F), but the cholesterol concentration of HDL-2 was not affected (Fig. 2E).

We determined the hepatic activities of a number of key enzymes in the synthesis and degradation of sphingomyelin. Rats fed the casein diet had greater activity of serine palmitoyltransferase on d 7 of the experiment, but this initial increase was transient (Fig. 3). Casein feeding for 7 d also significantly increased the activities of the sphingomyelin-synthesizing enzymes in hepatic plasma membranes, phosphatidylcholine:ceramide phosphocholinetransferase and phosphatidylethanolamine:ceramide phosphoethanolaminetransferase (Fig. 4). The activity of sphingomyelinase in the lysosomes was significantly reduced after 7 d of feeding the casein diet and that in the plasma membrane fraction was significantly raised (Fig. 5). Again, the casein-induced increases in enzyme activities were not seen after d 7 of the experiment.

**DISCUSSION**

Consistent with previous observations (Terpstra et al. 1982, Zhang et al. 1992), the rats fed the cholesterol-rich diet containing casein accumulated more cholesterol in liver and VLDL than did their counterparts fed soybean protein. The new finding is that consumption of casein caused a transient increase in the amount of sphingomyelin in the VLDL fraction. The increase on d 7 of the feeding trial was >60%. The casein-induced increase in VLDL-sphingomyelin was associated with decreases in the concentrations of sphingomyelin in LDL and HDL-2. The various changes in lipoprotein-sphingomyelin, including the transient nature of the changes, resulted in the outcome that dietary casein did not significantly affect sphingomyelin concentrations in whole plasma. In essence, the cholesterol and sphingomyelin concentrations in whole plasma and lipoprotein fractions showed similar time courses.

The amount of sphingomyelin in VLDL may reflect hepatic secretion of this phospholipid. An increased hepatic demand for sphingomyelin to be secreted with VLDL can be satisfied by...
increasing the rate of synthesis and/or uptake from circulating lipoproteins, and/or by decreasing the rate of catabolism. Determination of the activity of a number of key enzymes in the synthesis of sphingomyelin indicated that on d 7 of the experiment, the feeding of casein had increased the activities of serine palmitoyltransferase, phosphatidylcholine:ceramide phosphoethanolaminetransferase and phosphatidylethanolamine:ceramide phosphoethanolaminetransferase. On the other hand, the activity of acid sphingomyelinase, the key enzyme in the degradation of hepatic sphingomyelin, was significantly decreased on d 7. Taken together, the enzyme data suggest that the extra sphingomyelin in VLDL may result from an increase in the rate of hepatic formation and a decrease in catabolism. This statement implies that we assume that the observed decrease in the activity of lysosomal sphingomyelinase is more important with respect to overall hepatic sphingomyelin catabolism than is the observed increase in the activity of membrane sphingomyelinase. It is difficult to see that the increased demand for hepatic sphingomyelin in the rats fed casein is met by increased sphingomyelin uptake. The high hepatic cholesterol concentration in these rats will cause down-regulation of the number of LDL receptors (Brown and Goldstein 1981), which leads to reduced LDL uptake.

The casein-induced increase in VLDL-sphingomyelin after 7 d could relate to the exchange of sphingomyelin between lipoproteins. Plasma proteins capable of such transfer have been identified (Tall et al. 1985). When the concentration of

FIGURE 2 Cholesterol (panels A, C and E) and sphingomyelin (panels B, D and F) concentrations of VLDL, LDL and HDL-2 fractions isolated from rats fed diets containing either soybean protein or casein for up to 21 d. Samples of two rats were pooled; each value represents the mean ± SD of three pooled fractions. Significantly different from the diet containing soybean protein: aP < 0.05; bP < 0.02; cP < 0.001.

FIGURE 3 Effect of dietary casein vs. soybean protein on the hepatic activity of serine palmitoyltransferase. Each value represents the mean ± SD, n = 6. Per liver sample, enzyme assays were conducted in triplicate. Significantly different from the diet containing soybean protein: aP < 0.001.

FIGURE 4 Effect of dietary casein vs. soybean protein on the activities of phosphatidylcholine:ceramide cholinephosphotransferase (panel A) and phosphatidylethanolamine:ceramide ethanolaminephosphotransferase (panel B) in hepatic plasma membranes. Each value represents the mean ± SD, n = 6. Per liver sample, each enzyme assay was conducted in triplicate. Significantly different from the diet containing soybean protein: aP < 0.05; bP < 0.01.
VLDL-sphingomyelin was high in the casein-fed rats, the LDL- and HDL-2-sphingomyelin concentrations were low. This might be caused by sphingomyelin transfer between lipoproteins. However, the casein-induced fall in LDL-sphingomyelin, and also the lowering of group mean LDL cholesterol, may be caused by a decrease in the density of the LDL particles so that they are in part recovered in the VLDL fraction. Such a phenomenon explains the decrease in LDL cholesterol in cholesterol-fed rats (Beynen et al. 1984). Possibly, plasma transfer of sphingomyelin from HDL-2 to VLDL may contribute to the increase in VLDL-sphingomyelin in the casein-fed rats.

In conclusion, feeding of the casein-containing diet probably resulted in an increased output of sphingomyelin by the liver as reflected by raised levels of VLDL sphingomyelin. Sphingomyelin may be an essential structural component of VLDL (Merrill and Jones 1990) so that an increase in VLDL secretion, as occurs in casein-fed rats (Beynen 1990), is associated with increased sphingomyelin secretion. To meet the extra demand for sphingomyelin, the rate of sphingomyelin formation was enhanced by up-regulation of the activities of sphingomyelin-synthesizing enzymes, and the rate of its catabolism was lowered by down-regulation of the activity of lysosomal sphingomyelinase. Transfer of sphingomyelin from HDL-2 to VLDL particles might have contributed to the high level of VLDL-sphingomyelin in the casein-fed rats. The effects of casein feeding were transient, but the mechanism underlying the adaptive response to a change in sphingomyelin metabolism is unknown.

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LITERATURE CITED