Calcium in Milk Products Precipitates Intestinal Fatty Acids and Secondary Bile Acids and Thus Inhibits Colonic Cytotoxicity in Humans

Mirjam J. A. P. Govers, Denise S. M. L. Termont, John A. Lapréd, Jan H. Kleibeuker, Roel J. Vonk, and Roelof Van der Meer

Department of Nutrition, Netherlands Institute for Dairy Research (NIZO); P. O. Box 20, 6710 BA Ede (M. J. A. P. G., D. S. M. L. T., J. A. L., R. V. d. M.), and Departments of Internal Medicine [J. H. K.] and Pediatrics [R. J. V.]; University Hospital, 9713 EZ Groningen, the Netherlands

ABSTRACT

Dietary calcium may reduce the risk of colon cancer, probably by precipitating cytotoxic surfactants, such as secondary bile acids, in the colonic lumen. We previously showed that milk mineral, an important source of calcium, decreases metabolic risk factors and colonic proliferation in rats. We now report the effects of the habitual intake of milk calcium on metabolic risk factors in healthy subjects. A double-blind, cross-over metabolic study was performed in 13 healthy males. Placebo milk products (calcium, 3 mgl) were compared with regular milk products (calcium, 30 mgl). In each 1-week period, the habitual diet was recorded, and urine and feces were collected for 3 and 3 days, respectively. Milk calcium significantly increased fecal pH and fecal excretion of phosphate (132%), total fat (139%), free fatty acids (195%), and bile acids (141%), indicating intestinal complexation. In fecal water, the concentrations of long-chain fatty acids, secondary bile acids (deoxycholic and lithocholic acid), neutral sterols, and phospholipids were about halved (P < 0.05). Consistent with these changes in soluble hydrophobic surfactants, calcium decreased the cytotoxicity of fecal water from 68 ± 9 to 28 ± 12% (P < 0.005). Calcium in milk products precipitates luminal cytotoxic surfactants and thus inhibits colonic cytotoxicity. Therefore, habitual dietary calcium may contribute to a nutritional modulation of colon cancer risk.

INTRODUCTION

Sporadic colon cancer is a multifactorial disease probably affected by environmental determinants, such as the composition of the diet. Epidemiological studies indicate that the incidence of colon cancer is, for instance, positively associated with the intake of saturated fat (1, 2) and negatively associated with dietary calcium intake (3). To explain these associations, it has been hypothesized (see Ref. 4 for review) that dietary fat increases the intestinal concentrations of soluble fatty acids and secondary bile acids. These surfactants can damage the colonic epithelium and thus can stimulate the proliferation of colonic crypt cells (5, 6), which may increase the risk of colon cancer (7). Newmark et al. (8, 9) hypothesized that dietary calcium precipitates these soluble bile acids and fatty acids in the colonic lumen and thus inhibits their cytotoxic effects. Consequently, epithelial cell damage and epithelial proliferation are decreased. Since then, several in vitro studies (10–12), as well as animal (5, 6, 13–15) and clinical studies (see Refs. 16 and 17 for reviews), have demonstrated the different steps in the proposed mechanism of the antiproliferative effect of supplemental calcium on the colonic epithelium. Recently, the relevance of this mechanism was strongly supported by Steinbach et al. (18), who showed that the increased colonic concentrations of bile acids and fatty acids in intestinal bypass subjects are correlated with the hyperproliferation of rectal epithelial cells. Oral calcium carbonate supplementation inhibits this hyperproliferation.

Thus far, all human intervention studies concerning the mechanism of these protective effects of calcium have been performed by supplementing the habitual diet with pharmaceutical calcium preparations, such as calcium carbonate or gluconate (e.g., Refs. 19–21). Because of this design, effects of calcium are frequently studied against a background of a high habitual intake of dietary calcium, which may decrease the efficacy of the oral calcium supplements. Therefore, a quantitation of the possible protective effects of the habitual intake of dietary calcium is required. In Western diets, this habitual intake of calcium is mainly (about 70%) derived from milk and milk products. Therefore, we aimed to study the efficacy and mechanism of the protective effects of habitual dietary calcium by changing the calcium content of milk products. Using an analogous design, we demonstrated in rats that milk calcium precipitates cytotoxic surfactants, such as bile acids, fatty acids, and phospholipids, in the colonic lumen and thus inhibits the cytotoxicity of fecal water (22). These metabolic effects resulted in a drastic inhibition of colonic epithelial proliferation. To ascertain the relevance of these results for human intestinal physiology, we now report a placebo-controlled metabolic study in healthy volunteers. The mechanism of the possible protective effects of milk calcium was studied by comparing the differential effects of calcium-depleted and normal (calcium-rich) milk products on the fecal excretion of lipids and minerals and on the composition and cytotoxicity of fecal water.

MATERIALS AND METHODS

Subjects. Fifteen healthy male volunteers were recruited from the staff of the Netherlands Institute for Dairy Research. They were all without medication, including antibiotics, for at least 6 months. All subjects gave their informed consent, and all of them finished the study. From 2 subjects, it was hardly possible to prepare fecal water in the placebo period. The fecal water from these two subjects was extremely turbid and viscous and were thus difficult to analyze and not suitable for use in the cytotoxicity assay. Therefore, the data from these two subjects were not included in the study results, although their total fecal parameters did not differ from the others. The mean age (±SE) of the remaining 13 subjects was 38 ± 2 years, and mean body weight was 79 ± 3 kg.

Experimental Protocol. The study protocol was approved by the Medical Ethical Committee of the Wageningen Agricultural University (Wageningen, the Netherlands). The subjects were asked to maintain their usual dietary habits during the experimental period of 2 weeks, except that all liquid milk products were replaced by specially prepared milk products (see below) consumed during meals. A double-blind, cross-over design was used to control for time trends and carryover effects. The subjects were randomized into two groups consuming the experimental milk products in a different order. Each product was consumed for 1 week. During each 1-week period, the subjects recorded their intake of nutrients for 3 days (1 weekend day and 2 working days) in specially designed diaries. At the end of each period, all stool samples were collected for 3 days, and urine was collected for 1 day. All fecal and urinary samples were coded; therefore, the origins of the samples were unknown during processing and analysis. Body weights of the subjects were measured weekly.

Experimental Products. The experimental milk products (3% fat) differed only in calcium content and were manufactured by the Technology Department of the Netherlands Institute for Dairy Research. The placebo milk was prepared

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2. To whom requests for reprints should be addressed.

3270
from Calcitonin, a low-calcium whole milk powder (Nutricia, Zoetermeer, the Netherlands) and sterilized. This placebo milk contained 3 mm calcium. Regular milk powder was used to prepare the calcium-rich milk and contained 30 mm calcium. From both milks, yogurt was prepared by standard procedures, using cultures of Lactobacillus bulgaricus and Streptococcus thermophilus. Both products were coded to mask their origins during the study, and it appeared that subjects could not identify the placebo and regular products. The subjects were instructed to consume in total 1 liter/day milk and/or yogurt, according to their personal preference but similarly in both periods. Thus, these products provided 3 and 30 mmol/day (120 and 1200 mg/day) calcium in the placebo and calcium periods, respectively.

**Dietary Analyses.** The dietary records were coded for analysis by a dietitian, and nutrient intake was calculated from the computerized Netherlands food composition table, with adjustments for the experimentally prepared products.

**Urinary Analyses.** Creatinine was measured by a colorimetric assay (procured 555; Sigma Chemical Co., St. Louis, MO), and urea was measured by a colorimetric method, as described previously (23). Calcium was determined by atomic absorption spectrophotometry (model 1100; Perkin Elmer, Norwalk, CT), and phosphorus was determined as described by Fiske and Subbarow (24).

**Stool Collection and Fecal Water Preparation.** All stool samples were placed on dry ice immediately after defecation and subsequently stored at −20°C. After thawing overnight at 4°C, all samples of a subject, collected during 3 days, were pooled and homogenized. pH was measured at 37°C. Fecal water from fresh feces was obtained by centrifugation for 45 min at 24,000 × g, 37°C (Sorvall RC-5B; DuPont, Wilmington, DE). Fecal water was carefully aspirated, and pH was measured at 37°C. Samples were stored at −20°C until analysis. A part of the fecal homogenate was freeze-dried, and the percentage of dry weight was calculated. To study whether the cytotoxicity of fecal water was affected by the method of preparation, we also prepared fecal water from freeze-dried feces by reconstitution with double-distilled water to the original amount of water, as described previously (15, 21). After incubation for 1 h at 37°C in a shaking water bath, the samples were centrifuged for 30 min at 12,000 × g, 37°C (Hettich, Tuttingen, Germany). Fecal water was carefully aspirated and assayed for cytotoxicity.

**Total Feces Analyses.** After dry ashing of the freeze-dried feces, calcium and total phosphate were measured as described above. Inorganic phosphate was extracted from freeze-dried feces with 5% (w/v) trichloroacetic acid and measured as described above. Organic phosphate was calculated as the difference between total and inorganic phosphate. Total fat was determined according to the method of Henry et al. (25). Free fatty acids were extracted from freeze-dried feces with diethyl ether, as described previously (15), and measured using a colorimetric enzymatic assay (NEFA-C; Wako Chemicals, Neuss, Germany). Bile acids were extracted from freeze-dried feces with a tert-butanol:water mixture (1:1, v/v) and subsequently hydrolyzed and enzymatically quantitated exactly as described earlier (26).

**Fecal Water Analyses.** After acidification of fecal water with trichloroacetic acid (final concentration, 5%, w/v) and subsequent centrifugation (2 min at 10,000 × g, Eppendorf 5415), calcium and inorganic phosphate were measured in the supernatants as described above. Free fatty acids were determined as described previously (21). Briefly, fecal water supplemented with internal standards (heptanoic, tridecanoic, and heptadecanoic acids; Fluka, Buchs, Switzerland) was mixed with ethanol (1:1, v/v) and acidified with H2SO4 (final concentration, 0.5 M). Free fatty acids were extracted with diethyl ether:hexane (1:1, v/v), subsequently separated from other lipids on an aminopropyl column (Bond Elut; Varian, Harbor City, CA), and analyzed by gas chromatography (Mega 5160; Carlo Erba, Milan, Italy). A fused silica capillary column coated with Free Fatty Acid Phase (J & W Scientific, Folsom, CA) was used. The recovery of the individual C5–C20 fatty acids added to the fecal water was always greater than 95%. Bile acids and neutral sterols were identified and quantitated as described earlier (21). Briefly, fecal water supplemented with internal standards (7α,12α-dihydroxy-5β-cholic acid; Calbiochem, San Diego, CA; and 5α-cholestanol; Sigma) was hydrolyzed in methanol and NaOH at 80°C and subsequently extracted with petroleum ether to remove neutral sterols. After acidification with HCl (final concentration, 1 M), bile acids were extracted with diethyl ether. These extracts were methylated and silylated and then analyzed by capillary gas chromatography. The neutral sterols were determined in the petroleum ether extracts after silylation. The mean recoveries of added bile acids (deoxycholic and lithocholic acids) and cholesterol were 103 and 104%, respectively. Phospholipids were extracted from acidified fecal water (final concentration, 1 M HCl) using chloroform:methanol (27). After evaporation of chloroform under nitrogen, the phospholipids were digested with concentrated perchloric acid (15 min, 180°C) and quantitated as phosphate (28). Recoveries of added phosphatidylcholine and lysophosphatidylcholine were 100 and 89%, respectively.

**Cytotoxicity Assay.** Increasing volumes of fecal water (0–80 μl) were mixed with 154 mm NaCl to a total volume of 80 μl. After preincubation for 5 min at 37°C, 20 μl of a washed human erythrocyte suspension were added (final hematocrit, 5%) and incubated for 3 h at 37°C. The intact erythrocytes were precipitated by centrifugation for 1 min at 1,500 × g and washed twice with 154 mm NaCl, and after acidification with trichloroacetic acid (final concentration, 5%, w/v) and subsequent centrifugation (1 min at 10,000 × g), their potassium content was measured in the supernatant by atomic emission spectrophotometry (Perkin Elmer 1100). Simultaneously, erythrocytes were incubated in 154 mm NaCl (0% K+ release) and double-distilled water (100% K+ release). For each dilution of fecal water, the K+ release was calculated from these 0 and 100% controls. Cytotoxicity was quantitated as the area under the curve and expressed as a percentage of the maximal area, which implies 100% K+ release at each dilution of fecal water. We used this potassium measurement instead of our iron release method (15, 21), because we observed that the released iron precipitated during long-term incubations (>2 h) with turbid fecal water, which underestimates cytotoxicity. Probably, this precipitation is due to denaturation of the released hemoglobin in the turbid fecal water. It should be noted that, in a control experiment using clear fecal water, a high correlation was found between the two methods after a 3-h incubation (r = 0.95; n = 25; P < 0.001).

**Statistics.** Results are given as means ± SE; n = 13. Normally distributed differences between means were tested by the Student’s t test for paired samples. In other cases, the Wilcoxon signed rank test was used. Because our previous in vitro (10, 11) and animal studies (13–15), and human studies (21) have indicated that calcium decreases the concentration of hydrophobic surfactants in fecal water and thus its cytotoxicity, these data were tested for one-tail significance. Differences were regarded as significant if P < 0.05. Pearson’s (linear) correlation was used for regression analysis.

**RESULTS**

Table 1 shows that the dietary intake of the subjects during the study reflected a typical Western diet. No differences were seen between the placebo and calcium periods, except for daily calcium intake, which was 19.1 mmol (765 mg) during the placebo period and 45.5 mmol (1820 mg) during the calcium period. Body weights of the subjects slightly increased during the study (0.5 ± 0.2 kg), with similar weight gains in both periods. The urinary excretion of creatinine and urea did not differ between both periods (mean, 16.5 and 540 mmol/day, respectively). Calcium significantly increased the urinary excretion of calcium (from 3.6 ± 0.5 to 6.0 ± 0.7 mmol/day) and significantly decreased the urinary excretion of phosphate (from 50.3 ± 2.7 to 43.9 ± 2.5 mmol/day). Table 2 shows the total urinary plus fecal excretion of calcium and phosphate during the placebo and calcium periods. The daily phosphate excretion was similar in both periods, whereas the daily calcium excretion increased from 21.9 to

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Dietary intake of the subjects during the placebo and calcium periods</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
</tr>
<tr>
<td>Energy (megajoules/day)</td>
<td>13.1 ± 0.7a</td>
</tr>
<tr>
<td>Protein (energy, %)</td>
<td>13.8 ± 0.5</td>
</tr>
<tr>
<td>Fat (energy, %)</td>
<td>34.5 ± 2.0</td>
</tr>
<tr>
<td>Saturated</td>
<td>15.2 ± 0.7</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>12.9 ± 0.8</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>Carbohydrate (energy, %)</td>
<td>48.2 ± 2.2</td>
</tr>
<tr>
<td>Alcohol (energy, %)</td>
<td>3.0 ± 1.5</td>
</tr>
<tr>
<td>Fiber (g/day)</td>
<td>38.1 ± 3.6</td>
</tr>
<tr>
<td>Calcium (mmol/day)</td>
<td>19.1 ± 1.0</td>
</tr>
<tr>
<td>Phosphate (mmol/day)</td>
<td>75.8 ± 2.7</td>
</tr>
</tbody>
</table>

a Values are mean ± SE; n = 13. 
b P < 0.05, paired t test.
The integrity of extracted RNA was controlled by agarose gel analysis through the rRNA fraction.

CDNA synthesis was performed with 1 μg of total RNA in a 25-μl final volume containing 10 mM DTT, 50 mM Tris-HCL (pH 8.3), 75 mM KCl, 3 mM MgCl2, 300 μM dNTP, and 2.7 μM random hexamer oligonucleotides (Promega, Lyon, France). Samples were heated at 65°C for 5 min and rapidly chilled on ice before adding 40 IU of RNase inhibitor and 200 U of M-MTV reverse transcriptase (Life Technologies, Inc.). The final mixture was then incubated for 1 h at 37°C and heated for 5 min at 90°C. Reverse transcriptase efficiency was controlled by GAPDH housekeeping gene amplification, as described previously.

Competitive PCR. To competitively amplify the specific ER-α and -β gene fragments, the 5'-end oligonucleotide was identical for both PCRs (Fig. 1). To further minimize the differences in reaction efficiency between the two gene amplifications, the target nucleotide sequence was chosen in exons 3 and 4 displaying 71% homology. The 5' sense oligonucleotide was 5'-AAGAGCTGCCAGGCCTGCC-3' (ERxF4, located at nucleotides 702-720 and 454-472 of the ER-α and -β sequences, respectively). The 3' antisense oligonucleotide for ER-α was 5'-TGGCAGCTCTCATGTCTCC-3' (ERαR2, located at nucleotides 850-869), amplifying a 168-bp PCR product, whereas the 3' antisense oligonucleotide for ER-β was 5'-GCGCACTGGGGCGGCTGATCA-3' (ERβR1, located at nucleotides 701-721), generating a 267-bp specific fragment.

The reverse transcriptase product was amplified by PCR in a 25-μl final volume containing 20 mM Tris-HCL (pH 8.55), 16 mM (NH4)2SO4, 2.5 mM MgCl2, 10% DMSO, 50 μM of each dNTP (with 10 μM of oligonucleotides ERxF4, ERαR2, and ERβR1), and 0.125 unit of Taq DNA polymerase (Bioprobe, Montreuil, France). After an initial 2-min denaturation step at 94°C, PCRs were carried out on a DNA thermocycler under the following conditions: 1-min denaturation at 94°C, 1-min annealing at 62°C, and a 2-min extension at 72°C. The samples went a final extension step for 7 min at 72°C. Plasmids (pSG5) containing the wild-type full-length cDNA of ER-α and -β genes were used as DNA templates in the coamplification reaction. Each amplification product (12 μl) was

Table 3: Effects of calcium in dairy products on fecal parameters and fecal excretion of minerals.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Calcium</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stools/day</td>
<td>1.3 ± 0.1a</td>
<td>1.3 ± 0.1</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>Wet weight (g/day)</td>
<td>183 ± 22</td>
<td>218 ± 20</td>
<td>34 ± 11a</td>
</tr>
<tr>
<td>Dry weight (g/day)</td>
<td>44 ± 4</td>
<td>55 ± 4</td>
<td>11 ± 3b</td>
</tr>
<tr>
<td>pH</td>
<td>6.3 ± 0.1</td>
<td>6.6 ± 0.1</td>
<td>0.3 ± 0.1a</td>
</tr>
<tr>
<td>Calcium (mmol/day)</td>
<td>18.3 ± 2.0</td>
<td>43.1 ± 2.3</td>
<td>24.8 ± 19b</td>
</tr>
<tr>
<td>Total phosphate (mmol/day)</td>
<td>27.6 ± 2.9</td>
<td>36.5 ± 2.7</td>
<td>9.0 ± 1.5b</td>
</tr>
<tr>
<td>Inorganic</td>
<td>19.9 ± 2.4</td>
<td>24.7 ± 2.2</td>
<td>4.8 ± 1.2b</td>
</tr>
<tr>
<td>Organic</td>
<td>7.7 ± 1.0</td>
<td>11.8 ± 1.1</td>
<td>4.2 ± 1.3b</td>
</tr>
<tr>
<td>Fat (g/day)</td>
<td>6.7 ± 0.6</td>
<td>9.3 ± 0.7</td>
<td>2.6 ± 0.7b</td>
</tr>
<tr>
<td>Fatty acids (mmol/day)</td>
<td>8.7 ± 1.0</td>
<td>17.0 ± 2.3</td>
<td>8.3 ± 2.1b</td>
</tr>
<tr>
<td>Bile acids (μmol/day)</td>
<td>492 ± 68</td>
<td>692 ± 75</td>
<td>200 ± 40b</td>
</tr>
</tbody>
</table>

* Values are mean ± SE; n = 13.
* P < 0.05, paired t test.

49.1 mmol, which is in line with the recorded dietary intake (Table 1). The difference in daily calcium excretion between the two periods was 27.1 mmol/day, which reflects exactly the difference in contribution of the experimental products to the calcium intake (3 versus 30 mmol/day) and thus indicates excellent compliance with the study protocol.

First, we determined the effects of calcium in milk products on fecal parameters and fecal excretion of minerals and lipids, which are given in Table 3. Stool frequency was similar in both periods, whereas fecal output (wet as well as dry weight) and pH increased due to milk calcium. The extra amount of dietary calcium from the normal milk products was mainly (92%) excreted in the feces. Fecal phosphate excretion was also increased in the calcium period, due to a rise in both inorganic and organic phosphate excretion. The fecal excretion of fat was greater in the calcium period, indicating slightly decreased fat absorption (94.2 ± 0.5 and 92.8 ± 0.5% in the placebo and calcium periods, respectively; difference, -1.4 ± 0.6%; P < 0.05). In addition, fecal excretion of free fatty acids and bile acids was significantly increased in the calcium period.

Subsequently, we determined the composition of fecal water. The pH and mineral concentrations in fecal water are shown in Table 4. Consistent with the pH in feces, the pH of fecal water increased due to milk calcium. The pH values of feces and fecal water were highly correlated (r = 0.94; n = 26; P < 0.001). Whereas the calcium concentration did not change, the concentration of phosphate drastically decreased in the calcium period. The logarithm of the concentration product of calcium and phosphate was negatively correlated with the pH of fecal water (r = -0.73; n = 26; P < 0.001).

Fig. 1 shows the effects of milk on the total concentrations of surfactants in fecal water. Calcium nearly halved the concentrations of the ionic surfactants, free fatty acids, and bile acids, as well as those of the membranous lipids, cholesterol (and its bacterial metabolite coprostanol), and phospholipids. These calcium-dependent decreases were observed in all subjects, except one for fatty acids and phospholipids and another for phospholipids. Because the cytotoxicity of fatty acids and bile acids increases with their hydrophobicity (11, 29), it is important to quantitate the effects of calcium on the composition of these surfactants in fecal water. Fig. 2 shows that calcium did not affect the concentration of the nonlytic fatty acids C16-C18 and slightly decreased the low concentration of C15-C14 fatty acids. The decrease in total fatty acids was mainly (89%), caused by a decrease in the concentrations of the cytotoxic, long-chain fatty acids C16-C20. Especially, the concentration of stearic acid (C18:0) was drastically decreased. Fig. 3 shows that calcium did not affect the low concentrations of the primary bile acids cholic acid and chenodeoxycholic acid. Also, the concentrations of their hydrophilic metabolites 12-ketolithocholic acid and ursodeoxycholic acid were not changed. In contrast, the high concentrations of the secondary, hydrophobic, bile acids (iso)deoxycholic acid and (iso)lithocholic acid were significantly decreased by calcium. Consequently, the ratio of hydrophobic (mono and dihydroxy):hydrophilic (keto and trihydroxy) bile acids was decreased by calcium from 10.4 ± 2.1 to 5.9 ± 1.0 (P < 0.05).

Finally, we determined whether the observed changes in luminal surfactant concentrations affected the cytotoxicity of fecal water. Fig. 4 shows the individual values for cytotoxicity in the placebo and calcium periods. In 11 of 13 subjects, cytotoxicity was decreased by milk calcium, whereas in two subjects, cytotoxicity remained at 100% (these were the ones with deviating effects of calcium on fatty acid and/or phospholipid concentrations mentioned above). Overall, the cytotoxicity decreased...
from 68 ± 9% in the placebo period to 28 ± 12% in the calcium period (Wilcoxon, $P = 0.002$). In addition, we determined the cytotoxicity of fecal water that was prepared from freeze-dried feces. Analogous to the results described above, we found a decrease in cytotoxicity due to milk calcium: 60 ± 7% in the control period versus 32 ± 9% in the calcium period (Wilcoxon, $P = 0.001$). Moreover, values of fecal water prepared from fresh and freeze-dried feces were highly correlated ($r = 0.84$; $n = 26$; $P < 0.001$).

**DISCUSSION**

The present study shows for the first time that intrinsic dietary calcium has significant protective effects on luminal metabolic risk factors of colon cancer in healthy subjects. These effects can be attributed to milk calcium, because the dietary background of the volunteers was maintained constant during the study; there were no differences in the estimated dietary intake between the two periods (Table 1) or in the daily excretion of urea and phosphate. Moreover, the measured excretion of calcium was in line with the recorded dietary intake, and the difference in calcium content of the experimental products was completely recovered in urine and feces (Table 2). Taken together, these data indicate an excellent compliance with the study protocol.

Milk calcium increased the fecal excretion of phosphate and thus decreased urinary phosphate excretion (compare Tables 2 and 3). This indicates calcium-induced formation of insoluble calcium phosphate complexes in the intestine, as was shown previously (26, 30). Formation of intestinal insoluble calcium phosphate is further confirmed by the decreased phosphate concentration in fecal water and by the linear relationship between the pH of fecal water and the logarithm of the concentration product of calcium and phosphate, as found previously (21). Milk calcium increased the pH of feces and fecal water, which is also observed in studies using pharmaceutical calcium supplements (20, 21, 26, 31). This phenomenon was formerly attributed to the presence of equipotential amounts of carbonate in the calcium tablets; this argument, however, is not applicable in this study and, therefore, cannot explain the present increase in pH. Probably the calcium-dependent increase in fecal phosphate (Table 3) increases the fecal buffer capacity. Thus, acidification due to colonic fermentation may be buffered by the higher amount of phosphate present in the colonic lumen. This mechanism is consistent with the finding that dietary calcium stimulates the cecal fermentation of inulin and simultaneously increases cecal pH (32).

The increased fecal excretion of lipids (Table 3) already indicates that milk calcium precipitates luminal bile acids and fatty acids and thus inhibits their absorption. We previously showed that insoluble calcium phosphate binds and thus precipitates bile acids (10, 11). In line with these in vitro studies, we found that the extra amount of fecal phosphate and bile acids during the calcium period in healthy subjects was completely associated with calcium (26). Because of the similar increase in phosphate and bile acids, it is very likely that in the present study the bile acids are also precipitated by calcium phosphate.

Whether phosphate is involved in the precipitation of fatty acids by calcium (33) remains to be determined. In our analogous animal experiments (15, 22), the increased excretion of fatty acids due to dietary calcium was slightly counteracted by increasing dietary phosphate, suggesting that fatty acid precipitation also occurs via ionic binding to soluble Ca$^{2+}$ in the proximal small intestine. The present effect of milk calcium on fecal fatty acid excretion is greater than that of calcium carbonate supplementation of habitual diets containing high amounts of calcium (34, 35). This indicates that the efficacy of calcium to precipitate fatty acids decreases with increasing intake of calcium.

Whereas fecal excretion of fatty acids and bile acids increased, it should be stressed that their concentrations in fecal water were significantly decreased by milk calcium. This decrease in fatty acid concentration is mainly due to precipitation of fatty acids with chain lengths of 16 carbon atoms or more (Fig. 2). This effect is much greater than that observed after pharmaceutical calcium carbonate supplementation (21). We have shown before that these hydrophobic long-chain fatty acids are very cytotoxic (29). The present study shows for the first time that dietary calcium significantly decreases the total bile acid concentration in fecal water (Fig. 1), in contrast with our previous metabolic study using calcium carbonate tablets (21). In the latter study, however, the bile acid concentration was already below 200 $\mu$M in the control period, which was most likely due to the relatively high basal calcium intake (35 mmol/day) in that study. A further decrease by calcium was apparently not possible, probably because low concentrations of bile acid monomers do not bind to calcium phosphate (10, 11).

Dietary calcium predominantly precipitates the hydrophobic, secondary bile deoxycholic and lithocholic acids (Fig. 3). This is consistent with our finding that the hydrophobicity of bile acids is an important determinant of their binding to insoluble calcium phosphate.
(10, 11). Case-control studies show that levels of secondary bile acids, such as deoxycholic acid, are higher in serum and bile of adenoma subjects (36, 37), indicating that colon cancer risk is associated with a higher colonic exposure to, and absorption of, these bile acids. The epithelial hyperproliferation and increased colonic concentration of bile acids and fatty acids observed in intestinal bypass subjects (18) further corroborates this important association. Experimental studies show that secondary bile acids may promote colon carcinogenesis by several mechanisms. For instance, several studies suggest a direct stimulatory effect on epithelial cell proliferation (e.g., see Ref. 5). Alternatively, these bile acids may activate signal transduction pathways, resulting in increased eicosanoid production (38) and/or protein kinase C activation (39). In addition, these bile acids may also induce apoptosis resistance in immature colonic goblet cells (40). Thus, these clinical and experimental studies indicate that exposure of the colonic epithelium to secondary bile acids is an important etiological step in carcinogenesis. The significant precipitation of colonic deoxycholic and lithocholic acids observed in the present study shows that this metabolic risk factor can be decreased by habitual dietary calcium. Also, the observed decrease in concentrations of neutral sterols and phospholipids probably occurred because these lipids are solubilized as mixed micelles by hydrophobic ionic surfactants, such as bile acids. Whether these membranous lipids are solubilized from the colonic contents or directly from epithelial cell membranes is at present not known and requires further investigation.

The cytotoxicity of fecal water was drastically inhibited by milk calcium, which is consistent with the observed decrease in the concentrations of the hydrophobic fatty acids and bile acids. This is further supported by our in vitro study (29) showing that hydrophobic fatty acids combined with a mixture of deoxycholic and lithocholic acids (molar ratio, 3:1; similar to that in Fig. 3) have strong synergistic effects on cytotoxicity. Therefore, the combined results of our in vivo and in vitro studies indicate that the fecal water concentrations of these hydrophobic surfactants, but not their total fecal concentrations, are main determinants of luminal cytotoxicity. Caution should be taken in extrapolating the change in cytotoxicity of fecal water to proliferation of the colonic epithelium. We stress that our method for cytotoxicity is only a bioassay to quantitate the overall cell-damaging potency of the luminal contents, to which the colonic epithelium is exposed. The response of the colonic epithelium to these luminal changes depends on several “host-specific” factors, as discussed previously (21). However, lysis of erythrocytes has been shown to be similar to lysis of human colonic epithelial cells in vitro (29, 41), and in animal experiments, cytotoxicity of fecal water is highly correlated with colonic epithelial cell proliferation (14, 42). In our opinion, this indicates that the cytotoxicity of fecal water is a physiologically relevant intermediate step in the dietary modulation of colonic epithelial proliferation.

Taken together, the present study shows that calcium in milk products, within the normal range of habitual calcium intake, decreases the concentrations of hydrophobic fatty acids and secondary bile acids in fecal water. Consequently, the cytotoxicity of fecal water is drastically inhibited. Quantitatively, the extent of these effects of habitual dietary calcium is greater than that observed in studies using supranormal, pharmaceutical calcium supplementation. This indicates that these protective effects on metabolic risk factors are nonlinearly, probably sigmoidal, dependent on the amount of dietary calcium, provided other nutrients are maintained constant. This restriction is important, because we have shown earlier that the extent of the protective effects of calcium is dependent on the type and amount of dietary fat (14, 43). Qualitatively, the present results are similar to those of our recent animal study (22), in which we determined the mechanism of the antiproliferative effect of milk calcium. In that study, the decreased cytotoxicity resulted in decreased colonic epithelial proliferation. Because colonic epithelial proliferation is considered an important biomarker of the risk for colon cancer (7), the present study indicates that the habitual dietary intake of calcium can contribute to the nutritional modulation of colon cancer risk.

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