Mechanism of the Protective Effect of Supplemental Dietary Calcium on Cytolytic Activity of Fecal Water


Department of Nutrition, Netherlands Institute for Dairy Research (NIZO), PO Box 20, 6710 BA Ede, The Netherlands [J. A. L., H. T. De V., D. S. M. L. T., R. V. d. M.], and Department of Internal Medicine, University Hospital, Groningen, The Netherlands [J. H. K., E. G. E. De V.]

ABSTRACT

Dietary calcium supplementation inhibits hyperproliferation of rectal epithelium, possibly by precipitating luminal surfactants and thus preventing their cell-damaging effects. Therefore, we studied the effects of supplemental dietary calcium (35.5 mmol/day) on composition and cytolytic activity of fecal water and on the release of the epithelial marker alkaline phosphatase in 12 healthy volunteers. Fecal water was isolated by low-speed centrifugation. Cytolytic activity was determined as lysis of human erythrocytes by fecal water. Intestinal alkaline phosphatase activity in fecal water was measured with the use of the uncompetitive inhibitor L-phenylalanine. Supplemental calcium increased soluble calcium and decreased soluble Pi. The logarithm of the concentration product of calcium and phosphate was linearly dependent on pH. These observations indicate formation of insoluble calcium phosphate. Supplemental calcium did not alter the total bile acid concentration in fecal water but significantly decreased the ratio of more hydrophobic to more hydrophilic bile acids from 3.3 to 2.3. Calcium also significantly decreased the concentration of fatty acids (from 2.9 to 2.1 mmol/l). Consistent with these decreases in hydrophobic surfactants, calcium decreased the cytolytic activity of fecal water from 47 ± 9 to 27 ± 8% (n = 12, P < 0.05). Analogous to the decrease in cytolytic activity, the release of the epithelial marker alkaline phosphatase was also lowered by supplemental calcium. We conclude that supplemental dietary calcium decreases luminal cytotoxic surfactant concentrations and thus inhibits luminal cytolytic activity and the release of the epithelial marker alkaline phosphatase as an indicator of intestinal epitheliolysis. This mechanism may explain how dietary calcium could decrease epithelial cell proliferation.

INTRODUCTION

Epidemiological data indicate that colon cancer is associated with a high fat intake (1–3), probably by increasing the concentrations of soluble cytotoxic surfactants such as bile acids and fatty acids in the colon (1, 4, 5). Several epidemiological studies have also shown that dietary calcium is negatively associated with the risk of colon cancer (6, 7). It has been hypothesized that this effect is due to binding of soluble surfactants by calcium in the intestinal lumen (4, 5).

In vitro studies have shown that bile acids bind to insoluble calcium phosphate (8, 9) and that this binding decreases their well-known cell-damaging properties (9). In nutritional experiments with rodents, dietary calcium supplementation lowered colonic epithelial cell proliferation (10, 11). An increased colonic epithelial cell proliferation is considered to be a biomarker of an increased susceptibility to colon cancer (12). Studies by our group have indicated that in rats cytolytic activity of fecal water and colonic proliferation are highly correlated (13) and that dietary calcium supplementation lowers the concentration of soluble bile acids and luminal cytolytic activity (14, 15). One of our recent studies showed that in rats the effects of dietary calcium supplementation on soluble surfactants and luminal cytolytic activity are highly correlated with the Ca-dependent decreases in colonic epithelial proliferation (16).

Also, in humans, dietary calcium has been shown to reduce colonic cell proliferation (17–20). However, other studies have not found a protective effect of supplemental calcium (21–23). A number of human intervention studies have been conducted regarding the mechanism of the protective effects of dietary calcium. Interesting studies by Rafter et al. (24, 25), for example, showed effects of a low-risk (either low fat, high calcium, high fiber, or lactovegetarian) diet versus a high-risk (either high fat, low calcium, low fiber, or mixed) diet on cellular toxicity of fecal water. However, their study design, using complete diets with multiple dietary variables, could, of course, not provide information about the mechanism of the protective effects of calcium per se. Therefore, we studied the effects of supplemental calcium on the intestinal association of calcium, phosphate, and bile acids in a controlled study in humans (26). Analogous to the in vitro and animal experiments, we found that intestinal calcium, phosphate, and bile acids were closely associated. Results of in vitro (8, 9) and animal experiments (14–16) indicate that calcium decreases the concentrations of soluble bile acids and cytolytic activity of fecal water. No data are available to show whether these protective effects of dietary calcium also occur in humans. In order to elucidate the effects of supplemental calcium on cytolytic activity and composition of fecal water in humans, we determined these parameters in samples of our controlled study with healthy human volunteers (26). In addition, the release of the epithelial marker intestinal ALP as an indicator of intestinal epitheliolysis was studied.

MATERIALS AND METHODS

Experimental Procedures

Protocol and Participants. The protocol of the study and characteristics of its participants have been published (26). Briefly, 12 healthy male volunteers [age, 27.0 ± 1.2 years (mean ± SE); body weight, 75.1 ± 2.1 kg] participated in this study, which was approved by the Medical Ethical Committee of the University Hospital of Groningen. Their habitual dietary intake was registered by a dietician, and they were instructed to maintain a calcium-constant diet based on their own habitual diet. After a control period of 1 week, the subjects augmented their intake of calcium by ingesting tablets of calcium carbonate (made by the pharmacy of the University Hospital of Groningen) with their meals (total daily calcium carbonate, 35.5 mmol). This corresponds to 1440 mg/day of calcium and represents a doubling of their mean daily intake of calcium. In the Netherlands, the mean daily intake of calcium for this age group is about 1200 mg/day. Before and at the end of 1 week of calcium supplementation, the subjects collected feces for 3 days. Individual stool samples were stored in tightly closed boxes at 4°C immediately after defecation and handled within 12 h.

Preparation of Fecal Water. After the wet weight was determined, individual stool samples were weighed, homogenized in a Waring blender (Waring Products Division, New Hartford, CT), and subsequently freeze dried and weighed again. Fecal water was prepared by reconstituting individually pooled individual stool samples in water (10 min at 15,000 × g (Eppendorf 5415; Eppendorf Geratebau, Hamburg, Germany). The supernatant was carefully aspirated, and pH was measured at 37°C. Samples were stored at −20°C until further use. It should be noted that

3 The abbreviations used are: ALP, alkaline phosphatase; GC, gas chromatography; dsc, average film diameter; RCF, relative centrifugal force.
in rats fecal water obtained from freeze-dried feces using this procedure was not different from fecal water prepared from fresh feces with respect to cytolytic activity and to the concentrations of calcium, phosphate, bile acids, and fatty acids (13, 14).

Solubility Product Analyses. Na2HPO4 (final concentration, 8.0 mm) was added to solutions containing CaCl2 (final concentration, 20 mm) and MgCl2 (final concentration, 5.6 mm). These concentrations were chosen to mimic the relative amounts of these minerals in the feces of these volunteers (26). The tubes also contained buffer (final concentration, 100 mm) and NaCl to maintain a constant ionic strength of 150 mm. 2[N-morpholino]ethanesulfonic acid was chosen because fecal pigments may interfere with the spectrophotometric measurement of hemoglobin, these authors quantified hemolysis indirectly by counting the remainder of intact erythrocytes. Since we found that artifacts were produced by this method in control experiments (13), we used Fe release quantified by atomic absorption spectrophotometry as a measure of hemolysis. Human erythrocytes were isolated exactly as described previously (9). The incubation mixture contained 40, 80, 120, and 154 mm NaCl to a total volume of 160 µl and 40 µl cells (final hematocrit, 5%). In each assay, fecal water without erythrocytes was used to correct for the Fe content of the fecal water, and erythrocytes in double-distilled water (100% lysis) were used to determine the Fe content. Samples were incubated for 6 h at 37°C in a shaking water bath and then centrifuged for 1 min at 735 × g (Eppendorf 5415). This low value of RCF was chosen because control experiments showed that at higher RCF values hemoglobin occasionally coprecipitated; this was probably due to a denaturation of hemoglobin during the relatively long incubation time. With an RCF value of 735 × g, recovery of added hemoglobin was >95%. After the supernatants were centrifuged and diluted in double-distilled water, Fe content was measured using atomic absorption spectrophotometry. Cytolytic activity of each fecal water sample was quantified as presented in the lytic curve. This cytolysis activity is expressed as a percentage of the maximal area, which implies 100% lysis at each dilution of fecal water. The mean variation coefficient (SD/mean × 100%) of five different samples, each measured in triplicate, was 5.7%.

Intestinal ALP Activity Assay. Total ALP activity was determined according to the method of Bessey et al. (31) using a glycine buffer (final concentration, 100 mm, pH 9.8) in the presence of zinc (final concentration, 2 mm) and magnesium (final concentration, 5 mm). p-Nitrophenyl phosphate was used as the substrate, and the absorbance of the reaction product p-nitrophosphonic acid was determined spectrophotometrically at 405 nm. ALP activity was expressed as µmol p-nitrophosphonic acid/mg wet fecal sample (units/mg). Intestinal ALP activity was inhibited using 60 mm L-phenylalanine which acts as a specific competitive inhibitor of the intestinal isozyme (32). To validate the enzyme-kinetic inhibition, we compared this method with a method using immunoprecipitation. Immunoprecipitation was done using a commercially available rabbit antihuman intestinal polyclonal antibody (Dakopatts, Glostrup, Denmark) which is known for its cross-reactivity with the intestinal isozyme (33). Appropriate dilutions of human fecal water were incubated for 1 h at 37°C with antisera and rabbit control serum (Dakopatts) at the same protein concentrations in 154 mm NaCl + 3% polyethylene glycol 6000. After 16 h at 4°C, samples were centrifuged for 20 min at 15,000 × g (Eppendorf 5415), and in the supernatants of samples incubated with antisera or control serum, ALP activity was determined. Control experiments showed that after immunoprecipitation no inhibitory effect of 60 mm L-phenylalanine could be measured, which indicates that the intestinal isozyme has been completely precipitated by the antibody. This validation resulted in a nearly perfect correlation between the two methods (r = 0.98, n = 29, P < 0.001) (Fig. 1). Moreover, the regression equation (y = 1.0x − 0.01) indicated that both methods produced exactly the same values for intestinal ALP activities. Therefore, the enzyme-kinetic method was routinely used in the experiments.

Statistics. Values are given as means ± SE. Because our in vitro (9) and animal studies (14–16) have indicated that calcium decreases the hydrophobicity of surfactants and their cytolytic activity, differences between the control and calcium period were tested for one-tail significance. Differences in composition of fecal water were tested by Student’s t test for paired data. Data for cytolytic activity were tested using Wilcoxon’s signed-rank test. Differences were regarded as significant if P < 0.05. Data from the solubility product experiments were analyzed using regression analysis. The individual
DIETARY CALCIUM DECREASES CYTOLYTIC ACTIVITY OF FECAL WATER

RESULTS

The input-output analysis of this study was reported before (26). In summary, during the calcium supplementation period, the total (urinary + feces) output of phosphate and magnesium remained constant. Recovery of supplemental calcium was 95%, indicating an excellent dietary compliance. Calcium supplementation stimulated the fecal excretion of calcium, phosphate, and bile acids and slightly increased fecal pH.

In fecal water, the concentration of calcium was increased by supplemental calcium, whereas the concentration of soluble phosphate was drastically decreased (Fig. 2). This indicates the formation of insoluble calcium phosphate in the intestine (26). No effect was seen on soluble magnesium concentrations. Analogous to feces (26), the pH of fecal water was slightly increased by supplemental calcium.

In order to ascertain whether fecal soluble calcium and phosphate were in equilibrium with precipitated calcium phosphate, we quantified this equilibrium in vitro using mineral concentrations comparable with those in feces (26). As shown in Fig. 3, in vitro this equilibrium is reflected by a linear relationship between pH and the logarithm of the concentration product of calcium and phosphate in the equilibrated supernatants. This linear relationship for the solubility product versus pH is in accordance with the theory of calcium phosphate precipitation. Magnesium hardly coprecipitated with calcium and phosphate because >90% of the added magnesium was recovered in the supernatant. Also, for calcium and phosphate in fecal water, a linear relationship (r = -0.90) was observed, which paralleled the line for the solubility product of insoluble calcium phosphate in vitro.

In view of the hypothesis that dietary calcium inhibits luminal cytolytic activity by decreasing the concentrations of soluble surfactants, we quantified the bile acid and fatty acid concentrations in fecal water. The effects of supplemental calcium on the luminal surfactant concentrations are summarized in Table 1. The total bile acid concentration in fecal water was not altered by supplemental calcium. However, there was a trend toward a decrease in relatively hydrophobic and cytotoxic (9) mono- and dihydroxy bile acids and an increase in more hydrophilic and less cytotoxic (9) trihydroxy and keto bile acids. As a consequence the ratio of (mono + dihydroxy) to (keto + trihydroxy) bile acids is decreased (P < 0.01). Because the solubility of neutral sterols is dependent on the hydrophobicity of bile acids (34), we also determined the effects of supplemental calcium on cholesterol and coprostanol in fecal water. As can be seen in Table 1, the concentrations of cholesterol as well as of coprostanol were decreased by calcium supplementation.

Subsequently, we determined the effects of supplemental calcium on the fatty acid concentrations in fecal water. Total fatty acid concentration in fecal water was decreased by supplemental calcium. The most predominant fatty acids in fecal water were C6, C8, C16, C18:0, and C18:1, with only trace amounts of the other fatty acids (Table 1). None of the individual fatty acids except for C18:1 showed a significant reduction in concentration after calcium supplementation.

Subsequently, we analyzed whether the observed decrease in potentially cytotoxic surfactants resulted in a lower cytolytic activity of fecal water. In 11 of the 12 persons, cytolytic activity of fecal water was decreased by supplemental dietary calcium (Fig. 4). Cytolytic responses in cytolytic activity and ALP activity were correlated by Spearman’s rank correlation test.
Table 1  Effects of supplemental dietary calcium on bile acid composition, neutral sterols, and fatty acid composition in fecal water

<table>
<thead>
<tr>
<th></th>
<th>Control (μM)</th>
<th>Calcium (μM)</th>
<th>Difference (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bile acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithocholate</td>
<td>175 ± 58a</td>
<td>205 ± 93</td>
<td>30 ± 38</td>
</tr>
<tr>
<td>Iso lithocholate</td>
<td>11 ± 2</td>
<td>6 ± 1</td>
<td>−5 ± 1b</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>5 ± 1</td>
<td>1 ± 1</td>
<td>−4 ± 1c</td>
</tr>
<tr>
<td>Isodeoxycholate</td>
<td>61 ± 15</td>
<td>55 ± 14</td>
<td>−5 ± 4</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>18 ± 3</td>
<td>18 ± 4</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>12-Ketolithocholate</td>
<td>4 ± 1</td>
<td>6 ± 2</td>
<td>2 ± 1d</td>
</tr>
<tr>
<td>Cholate</td>
<td>26 ± 10</td>
<td>28 ± 10</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>Cholate</td>
<td>13 ± 4</td>
<td>27 ± 13</td>
<td>14 ± 10</td>
</tr>
<tr>
<td>Ketodihydroxy cholanate</td>
<td>7 ± 5</td>
<td>23 ± 20</td>
<td>16 ± 16</td>
</tr>
<tr>
<td>Others</td>
<td>31 ± 21</td>
<td>41 ± 33</td>
<td>10 ± 12</td>
</tr>
<tr>
<td>Ratio (mono + dihydroxy) to (keto + trihydroxy) bile acids</td>
<td>3.3 ± 0.5</td>
<td>2.3 ± 0.4</td>
<td>−1.0 ± 0.3c</td>
</tr>
<tr>
<td><strong>Neutral sterols</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>101 ± 16</td>
<td>59 ± 8</td>
<td>−42 ± 10b</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>50 ± 10</td>
<td>34 ± 5</td>
<td>−15 ± 5c</td>
</tr>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6-C8</td>
<td>2863 ± 611</td>
<td>2126 ± 374</td>
<td>−737 ± 272c</td>
</tr>
<tr>
<td>C16</td>
<td>2254 ± 580</td>
<td>1733 ± 328</td>
<td>−521 ± 365</td>
</tr>
<tr>
<td>C18</td>
<td>156 ± 28</td>
<td>112 ± 22</td>
<td>−44 ± 27</td>
</tr>
<tr>
<td>C18:1</td>
<td>149 ± 32</td>
<td>104 ± 38</td>
<td>−45 ± 45</td>
</tr>
<tr>
<td>Minor fatty acids</td>
<td>115 ± 64</td>
<td>97 ± 76</td>
<td>−18 ± 26</td>
</tr>
</tbody>
</table>

a Values are means ± SE, n = 12.
b p < 0.01.
c p < 0.001.
d p < 0.05.

activity was increased by extra calcium in only one person. This person also had a decrease in total fecal and fecal water pH, whereas the other persons had an increase in fecal water pH after calcium supplementation. Cytolytic activity was decreased (P < 0.025, Wilcoxon) by supplemental calcium from 47 ± 9% (median, 41%) to 27 ± 8% (median, 21%) for these 12 volunteers (for n = 11, calcium decreased cytolytic activity from 50 ± 9 to 23 ± 7%).

To extend these observations concerning the cytolytic activity of fecal water, we determined whether the protective effects of supplemental calcium on cytolytic activity are reflected in effects on the release of the epithelial marker alkaline phosphatase as a possible indicator of intestinal epithelialysis. This marker, measured as its activity in fecal water, showed effects similar to the effects on cytotoxicity (Fig. 4). Supplemental calcium decreased the activity of intestinal ALP from 1.44 ± 0.29 units/ml (median, 1.15 units/ml) to 0.81 ± 0.19 units/ml (median 0.70 units/ml). Consistent with the increase in cytolytic activity of fecal water observed in one person after calcium supplementation, the activity of intestinal ALP also increased in this person during the supplemental calcium period. The individual response in cytolytic activity to calcium supplementation was correlated with the response in ALP activity using Spearman’s rank correlation test, and this resulted in a significant association with a correlation coefficient of 0.63 (P < 0.02).

**DISCUSSION**

The results of this study concerning the effect of supplemental calcium on fecal water composition are consistent with the effect on intestinal association of calcium, phosphate, and bile acids observed in the first part of the study (26). For instance, pH of feces (26) and of fecal water slightly increased after CaCO3 supplementation, which is probably due to the use of carbonate. The decrease of the phosphate concentration in fecal water is consistent with the increase in precipitated phosphate concentrations in feces (26). Combined with the calcium phosphate precipitation experiment, these results strongly suggest the formation of insoluble calcium phosphate in the intestine, as was shown in nutritional experiments with rats (15). It should be noted that the intercept of the regression line for fecal water is different from that for calcium phosphate precipitation in vitro (Fig. 3).

Thus, the concentrations of soluble calcium and phosphate are higher in feces than those observed in vitro. These high concentrations of soluble calcium, magnesium, and phosphate have also been found in studies using in vivo dialysis (35). This can be explained by the lower activity coefficients for calcium and phosphate in fecal water compared to the in vitro conditions. This is probably due to the higher viscosity of fecal water, but this requires further investigation.

Supplemental calcium did not affect the low total concentration of bile acids in fecal water, which is in accordance with analogous results
also not precipitated by calcium phosphate (8, 9). In contrast to the ratio of hydrophobic to hydrophilic bile acids was also low-er. With regard to this effect, it should be noted that our in vitro studies indicate that a decreased hydrophobicity of bile acids inhibits their cytolytic activity (9). Bile acids are not the only surfactants in fecal water; therefore, we also determined the soluble free fatty acid concentrations. The decrease in soluble fatty acids that we found after supplemental calcium cannot be directly compared with the concentra-tions observed in the multivariate studies of Raft et al. (24, 25). Moreover, their method of determination of fatty acids did not dis-tinguish between the (cytolytic) free fatty acids and those derived from tri-, di-, and monoglycerides and of phospholipids. Conse-quently, the concentrations given by these authors are higher than the concentrations shown in Table 1. In line with the decrease in free fatty acid concentration and the shift from hydrophobic to hydrophilic bile acids in fecal water, a decrease in neutral sterol concentration was observed. These changes in fecal water lipids show that supplemental calcium causes a decrease in hydrophobic components of fecal water.

The observed calcium-dependent decreases in fatty acid concentra-tion and in hydrophobicity of the bile acids during supplemental calcium resulted in a decreased cytolytic activity of fecal water. This effect of calcium is consistent with the effects observed in our in vitro studies (37). In those studies, we showed that fatty acids with a carbon number >10 are cytolytic to erythrocytes and that bile acids in concentra-tions measured in fecal water synergistically stimulate this fatty acid-induced cytolytic activity. This synergistic effect increases with increasing hydrophobicity of the bile acids (37).

The membrane-damaging activity of bile acids and fatty acids is a well-established intrinsic physicochemical property of these surfac-tants. Lysis, however, is determined by this cytolytic activity, as well as by the susceptibility of the plasma membrane exposed to these surfactants. For this reason, we wish to stress that our hemolysis assay is only meant to quantify the calcium-dependent changes in overall cytolytic activity of the luminal surfactants to which the colonic epithelium is exposed. Our data should not be interpreted as a quantitative measure of epitheliolysis caused by these surfactants because the determinants of the epithelial susceptibility are at present largely unknown. For instance, individual differences in composition and thickness of the protective mucin layer as well as in lipid composition of the apical membrane of the colonocyte (38) may modulate the epithelial susceptibility to luminal surfactants. Recent experiments indicate, however, that surfactant-induced lysis of erythrocytes (9, 37, 39) is similar to that of human colonic epithelial cells in vitro (37, 40). In addition, it should be noted that diet-induced changes of cytolytic activity of fecal water and of in vivo colonic epithelial cell proliferation are highly correlated in rats (13). This at least indicates that the cytolytic activity of fecal water is a physiologically relevant intermediate in the mechanism of dietary modulation of colonic proliferation.

This is further supported by recent work from our laboratory which shows that release of the epithelial exoenzyme alkaline phosphatase reflects epitheliolysis (37). Moreover, in animal studies, the release of ALP was highly correlated with luminal cytolytic activity as well as with in vivo colonic proliferation (41), suggesting cause-and-effect relationships. In the present study with human volunteers, we quan-titated the release of this epithelial marker by measuring the activity of intestinal ALP in fecal water. Analogous to the decrease in cytolytic activity after calcium supplementation, the activity of intestinal ALP was decreased by calcium supplementation, which indicates that the decrease in cytolytic potency of the intestinal contents lowered intesti-nal epitheliolysis.

These protective effects of supplemental dietary calcium on com-position, cytolytic activity of luminal surfactants, and release of the epithelial marker ALP offer a molecular explanation for the calcium-dependent inhibition of epithelial hyperproliferation in patients at risk for colon cancer (17–20). As mentioned above, this mechanism is consistent with in vitro studies on solubility and cytotoxicity of bile acids and fatty acids (8, 9) and with the protective effect of calcium observed in animal studies (14–16). To investigate whether the protective effects of calcium on luminal cytolytic activity and intestinal epitheliolysis observed in healthy human volunteers can also be found in patients at high risk for colon cancer, additional combined clinical and biochemical studies are warranted.

ACKNOWLEDGMENTS

The authors wish to thank C. De Jong, R. Poelstra, R. Neeter, and H. Teisman for their assistance with the GC/mass spectrometer analysis.

REFERENCES


Mechanism of the Protective Effect of Supplemental Dietary Calcium on Cytolytic Activity of Fecal Water
