The Antiproliferative Effect of Dietary Calcium on Colonic Epithelium Is Mediated by Luminal Surfactants and Dependent on the Type of Dietary Fat

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ABSTRACT

Bile acids and fatty acids may promote colon cancer by inducing colonic hyperproliferation. Dietary calcium inhibits the promoting effects of bile acids and fatty acids, possibly by precipitating these surfactants and lowering their cytotoxic activity. Because bile acids and fatty acids are products of fat digestion, their effects may be dependent on the type of dietary fat. The effects of the type of dietary fat (energy percentage, 40) and of CaHPO₄ supplementation (25 versus 225 µmol/g diet) on the luminal solubility of surfactants, cytotoxic activity, epitheliolysis, and in vivo colonic proliferation were studied in rats using Western high-risk diets. The different types of commercially available fats were butter, saturated margarine, and polyunsaturated margarine. Supplemental calcium drastically increased fecal fatty acid excretion, the effect being dependent on the type of fat, and slightly stimulated fecal bile acid excretion. Soluble surfactant concentrations were drastically decreased by calcium supplementation with all three types of dietary fat. Consequently, cytotoxic activity of fecal water was decreased by supplemental calcium. These luminal effects of calcium resulted in a lower intestinal epitheliolysis. The compensatory proliferation of the colonic epithelium was decreased by supplemental CaHPO₄ for the butter and saturated margarine diets. Despite CaHPO₄-dependent decreases in luminal effects and epitheliolysis, no significant decrease in proliferation on the polyunsaturated margarine diet was observed. Multiple regression analysis of soluble surfactants with cytotoxic activity (R = 0.76), epitheliolysis (R = 0.74), and colonic proliferation (R = 0.84) showed highly significant associations. Cytotoxic activity and epitheliolysis as well as epitheliolysis and proliferation were highly correlated (r = 0.97 and r = 0.88, respectively; n = 36) for control and CaHPO₄-supplemented diets, suggesting cause-and-effect relationships. It is concluded that the antiproliferative effect of dietary calcium is mediated by the precipitation of luminal surfactants and is dependent on the type of dietary fat.

INTRODUCTION

Several types of epidemiological studies have reported positive associations between the incidence of colon cancer and the dietary intake of fat (1–3). Negative correlations have been found for a high calcium intake and colon cancer (4, 5).

Tumor induction studies with rodents have shown that bile acids and fatty acids may act as promoters/carcinogens (6–8). One important mechanism for this promotive effect is the induction of colonic hyperproliferation, which can be considered to be a biomarker of an increased susceptibility to colon cancer (9). Several studies using a semiphysiological design with intrarectal instillation of bile acids or fatty acids in rodents have shown that these surfactants may indeed induce higher proliferation rates in the colon (10–12). Simultaneous administration of calcium reduces these hyperproliferative effects (11, 12).

With regard to the mechanism of the promotive effect of dietary fat and the protective effect of dietary calcium, Newmark et al. (13) have proposed that a high-fat diet raises the concentrations of potentially cytotoxic bile acids and fatty acids in the colon. Damage of the colonic epithelium by these surfactants is then compensated for by an increased epithelial proliferation. Calcium may form complexes with the cytotoxic surfactants in the intestinal lumen thus reducing their cytotoxic and hyperproliferative effects (13). Several lines of experimental evidence now support this proposed sequence of effects. In vitro studies have shown that bile acids and fatty acids are toxic to different types of cells (14–19). Bile acids are bound to calcium phosphate (18, 20, 21), which reduces their cytotoxic activity (18). The cytotoxic activity of fatty acids is blocked by calcium (16). Nutritional studies with rodents have demonstrated that a diet-induced increase in colonic surfactant concentrations stimulates cytotoxic activity as well as colonic proliferation (22). Dietary CaHPO₄ decreases concentrations of soluble surfactants and the cytotoxic activity of fecal water (23). Proliferation induced by feeding of bile acids is also decreased by supplemental dietary CaHPO₄ (24, 25). In recent studies Newmark et al. (26) showed that a diet high in fat and low in calcium and vitamin D induced higher proliferation rates compared to a control diet. In humans, the cytotoxic activity of fecal water is lowered by supplemental calcium (27), as is proliferation of colonic epithelium (28, 29). However, the different steps in the mechanism (i.e., effects on solubility of surfactants, luminal cytotoxic activity, intestinal epitheliolysis, and the compensatory proliferation of the colonic epithelium), as far as we know, have never been quantified in one study. In the present study we investigated this proposed sequence of effects in rats fed Western high-risk diets (control) or diets supplemented with CaHPO₄. We used three different types of dietary fat, mimicking a human diet with a high content of saturated medium-chain triglycerides (butter), a diet with a high content of long-chain saturated fat (saturated margarine), and a diet with a high content of PUFA (polyunsaturated fatty acids).

MATERIALS AND METHODS

Animals and Diets. Eight-week-old male outbred Wistar rats (Small Animal Research Center of the Wageningen Agricultural University) (body weight, 192 g) were housed individually at a constant temperature of 21°C. During the experimental period of 2 weeks, groups of rats (6 rats/group) were fed a purified diet which differed in CaHPO₄ content (25 and 225 µmol/g diet) and in type of dietary fat (AIN-76A diet contains 130 µmol Ca/g diet). The calcium content of the control diet (25 µmol/g diet) is comparable to a human daily intake of 500 mg Ca/day, whereas the recommended dietary calcium intake for adults is 800 mg/day. Three types of commercially available dietary fats were used: butter, saturated margarine, and a margarine with a high content of polyunsaturated fatty acids. The fatty acid composition of the experimental diets measured by gas chromatographic analysis according to the method of Badings and De Jong (30) is given in Table 1. Correction was made for the cholesterol content of the butter and saturated margarine diets by supplementing the PUFA-margarine diet with 0.05% (wt/wt) cholesterol (BDH Chemicals, Ltd., Poole, England). The composition of the low-CaHPO₄ diet was (g/kg diet): casein (acid casein; DMV Veghel, the Netherlands), 200; dextrose monohydrate (AVEBE, Foix, the Netherlands), 460; corn oil (Reddy, Rotterdam, the Netherlands), 20; butter (Super Select B.V., Utrecht, the Netherlands), 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: ALP, alkaline phosphatase; PUFA, polyunsaturated fatty acids.

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saturated margarine (Van den Bergh en Jurgens B.V., Rotterdam, the Netherlands), or polyunsaturated margarine (Van den Bergh en Jurgens B.V.), 215; cellulose (ENKA B.V., Arnhem, the Netherlands), 20; mineral mix, 35; vitamin percentage; 20% wt/wt). low fiber, and low calcium. Feed and water were supplied ad libitum. Animal weights were recorded weekly, and feed intake was measured every 2 days. Feces were collected quantitatively during days 11-14 of the experiment.

**Table 1** Fatty acid composition of the diets containing butter, saturated margarine, or PUFA-margarine (μmol/g diet)

<table>
<thead>
<tr>
<th>Saturated</th>
<th>Butter</th>
<th>Saturated margarine</th>
<th>PUFA margarine</th>
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<tbody>
<tr>
<td>C16:0</td>
<td>160</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>C17:0</td>
<td>33</td>
<td>68</td>
<td>14</td>
</tr>
<tr>
<td>C18:0</td>
<td>84</td>
<td>171</td>
<td>68</td>
</tr>
<tr>
<td>C18:1</td>
<td>65</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>C20:0</td>
<td>3</td>
<td>51</td>
<td>7</td>
</tr>
<tr>
<td>Monoensaturated</td>
<td>13</td>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td>C18:1</td>
<td>165</td>
<td>158</td>
<td>140</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>47</td>
<td>111</td>
<td>431</td>
</tr>
<tr>
<td>C18:2</td>
<td>47</td>
<td>22</td>
<td>4</td>
</tr>
</tbody>
</table>

Total ALP activity was determined according to the method of Bessey et al. (35) using a glycine buffer (final concentration, 100 mM; pH 9.8) in the presence of ZnSO₄ (final concentration, 2 mM) and MgCl₂ (final concentration, 5 mM). p-Nitrophenylphosphate was used as the substrate, and the absorbance of the reaction product p-nitrophenol was determined spectrophotometrically at 405 nm. ALP activity was expressed as μmol p-nitrophenol/min/ml fecal water (units/ml). Intestinal ALP activity was inhibited using 60 μl 1-phenylalanine, which acts as a specific competitive inhibitor of the intestinal isoform in humans and rats (36). The difference between the total (noninhibited) activity and the activity after inhibition with 1-phenylalanine is the activity of the intestinal isoform. This enzyme-kinetic measurement of intestinal ALP in fecal water correlated highly (r = 0.98; y = 1.03 × −0.01) with the immunoprecipitation method for determining intestinal ALP (37).

**Statistics.** Values are the means of six rats with their S.E. After analysis of variance the differences between the means of the groups were tested using Fisher's protected least significant difference test (two-sided). Differences were regarded as significant if P < 0.05. Data comparing cytolytic activity of fecal water with intestinal epithelialysis and epithelialysis with colonic proliferation were analyzed by single linear regression analysis. Multiple regression analysis of the effect of luminal surfactants on cytolytic activity, epithelialysis, and proliferation was done using a commercially available statistical package (SPSS/PC+ v2.0) (SPSS, Inc., Chicago, IL).

**RESULTS**

Feed intake (19.1 ± 0.2 g/day) and weight gain (79.9 ± 1.6 g/14 days) were not significantly affected by the experimental diets. Supplemental CaHPO₄ significantly increased fecal mass (g dry/day) on the saturated margarine diet and stimulated the fecal excretion of calcium and inorganic phosphate in all of the diets (Table 2). No significant effects of the experimental diets on the pH of fecal water were observed. Concentrations of calcium and inorganic phosphate in fecal water were not significantly different between the different types of dietary fat. Supplemental CaHPO₄ significantly increased both the concentrations of calcium and inorganic phosphate in fecal water on all three types of dietary fat (Table 2).

Total free fatty acid concentration was significantly lower on the PUFA-margarine control diet compared to the butter and saturated-margarine control diets (Fig. 1). The free fatty acid excretion was drastically increased by supplemental CaHPO₄ with the same fat-type dependency. Supplemental CaHPO₄ slightly increased the total fecal concentration of bile acids (Fig. 1).

Because the cytolytic effects of surfactants are not mediated by their total fecal concentrations (23), we quantified the concentrations of fatty acids and bile acids in fecal water (Fig. 2). In contrast to the increases in total fecal fatty acids, the concentrations of soluble fatty acids were drastically decreased by supplemental CaHPO₄. Analogous to the total fatty acid concentrations, concentrations of soluble fatty acids were the lowest on the PUFA-margarine diets (Fig. 2). The
concentrations of fatty acids and bile acids are important determinants of cytolytic activity (R = 0.76).

Luminal cytolytic activity as measured by lysis of erythrocytes reflects the potency of the colonic contents in damaging cells and could result in changes in intestinal epithelioysis. Intestinal ALP activity in fecal water might reflect intestinal epithelioysis (17, 37), and therefore we determined the release of this epithelial marker as a measure of intestinal epithelioysis. The fat-type-dependent effects on luminal surfactants and cytolytic activity are reflected in effects on intestinal epithelioysis. The calcium-dependent decreases in soluble surfactant concentrations and cytolytic activity resulted in comparable decreases in intestinal epithelioysis (Fig. 3). Concentrations of soluble surfactants and intestinal epithelioysis were shown to be highly correlated by multiple regression analysis (R = 0.74). Subsequently we investigated whether the effects on intestinal epithelioysis resulted in changes in the compensatory proliferation of colonic epithelium. For the butter and the saturated margarine diets, the calcium-dependent decreases in luminal surfactants, luminal cytolytic activity, and intestinal epithelioysis resulted in a lower colonic proliferation, but no significant decrease in colonic proliferation on the PUFA-margarine diet was observed. Multiple regression analysis showed that luminal surfactants and in vivo colonic proliferation were highly correlated (R = 0.84). In contrast, no significant associations were found between the concentrations of calcium in fecal water and colonic proliferation.

Because the hypothesis of Newmark et al. (13) predicts causal relationships between luminal cytolytic activity, intestinal epithelioysis, and colonic epithelial proliferation, we correlated the data for the individual rats (Fig. 4). Luminal cytolytic activity, reflecting the potency of the colonic contents to damage cells, is highly correlated (r = 0.97; n = 36; P < 0.001) with intestinal epithelioysis for control diets as well as CaHPO4-supplemented diets. Intestinal epithelioysis is also highly correlated with colonic proliferation (r = 0.88; n = 36; P < 0.001) for control and CaHPO4-supplemented diets.

**DISCUSSION**

Supplementation of Western high-risk diets with CaHPO4 resulted in a drastic increase in total fatty acids and slight increases in total bile acid concentrations in feces. In contrast, the concentrations of soluble surfactants were drastically decreased by supplemental CaHPO4. Thus, CaHPO4 supplementation resulted in an increased precipitation of hydrophobic surfactants consistent with the first step in the hypothesis of Newmark et al. (13). The precipitation of fatty acids is probably caused by calcium-fatty acid soaps (38). Whether phosphate is involved in this complexation (39, 40) is at present not known and requires further investigation. The lower total concentration of fecal fatty acids on the PUFA-margarine diet compared with the other two diets might be caused by an impaired interaction of (poly)unsaturated fatty acids with calcium. Another possibility might be that calcium soaps of (poly)unsaturated fatty acids are more soluble in the intestinal environment (41) and may therefore be better absorbed compared to

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**Table 2: Effects of type of dietary fat and supplemental CaHPO4 on fecal mass and fecal excretion and fecal water concentrations of calcium and inorganic phosphate**

Control diets contain 25 μmol CaHPO4/g diet, and diets with supplemental calcium contain 225 μmol CaHPO4/g diet. Values are means of six rats ± SEs. Values in the same row not sharing the same superscript are significantly different: P < 0.05 (Fisher’s protected LSD test).

<table>
<thead>
<tr>
<th></th>
<th>Butter</th>
<th>Saturated margarine</th>
<th>Polyunsaturated margarine</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+Calcium</td>
<td>Control</td>
</tr>
<tr>
<td>Fecal mass (g dry/day)</td>
<td>1.25 ± 0.08&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.32 ± 0.10&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.09 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Calcium (μmol/g dry)</td>
<td>33 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2140 ± 90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36 ± 11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inorganic phosphate (μmol/g dry)</td>
<td>46 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1330 ± 80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fecal water pH</td>
<td>7.7 ± 0.1</td>
<td>7.3 ± 0.2</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>Calcium (mm)</td>
<td>0.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inorganic phosphate (mm)</td>
<td>3.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.7 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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Fig. 1. Concentrations of total free fecal fatty acids (FA) and of total fecal bile acids (BA) (μmol/g dry weight) of rats fed diets differing in type of dietary fat and amount of CaHPO4. Values are means of six rats ± SEs. Bars not sharing the same superscript are significantly different: P < 0.05. Control diets contain 25 μmol CaHPO4/g diet, and diets with supplemental calcium contain 225 μmol CaHPO4/g diet.
calcium in the colonic lumen is predominantly present as insoluble calcium phosphate (42–44).

Recently we have shown that effects of diet on colonic proliferation could be mediated by soluble surfactant concentrations and luminal cytolytic activity (22). Supplemental calcium lowers proliferation induced by bile acids or fatty acids in rodents when instilled intrarectally (11, 12) or added to the diet (24, 25). Our present study investigated the combination of luminal effects of CaHPO4 supplementation and the subsequent response of colonic epithelium. The lack of association between concentrations of soluble calcium and colonic proliferation indicates that luminal calcium concentrations have no major direct effect on in vivo colonic proliferation in contrast to results obtained in vitro (15, 45, 46). However, concentrations of fatty acids and bile acids were highly correlated with in vivo colonic proliferation, suggesting that these surfactants are important determinants of colonic proliferation. Consistent with the hypothesis of Newmark et al. (13), the intermediate steps between soluble surfactants and colonic proliferation consist of cytolytic effects of the intestinal contents resulting in intestinal epitheliolysis.

The luminal effects of dietary CaHPO4 supplementation, i.e., a decrease in soluble surfactants and luminal cytolytic activity, resulted in a decreased intestinal epitheliolysis. For the butter and saturated margarine diets, these effects of supplemental CaHPO4 caused a lower proliferation consistent with the proposed sequence of effects. How-

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**Fig. 2.** Concentrations of free fatty acids (FA) and bile acids (BA) in fecal water (mm) and cytolytic activity of fecal water of rats fed diets differing in type of dietary fat and amount of CaHPO4. Values are means of six rats ±SEs. Bars not sharing the same superscript are significantly different: P < 0.05. Control diets contain 25 μmol CaHPO4/g diet, and diets with supplemental calcium contain 225 μmol CaHPO4/g diet.

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**Fig. 3.** Intestinal epitheliolysis and colonic epithelial proliferation of rats fed diets differing in type of dietary fat and amount of CaHPO4. Values are means of six rats ±SEs. Bars not sharing the same superscript are significantly different: P < 0.05. Control diets contain 25 μmol CaHPO4/g diet, and diets with supplemental calcium contain 225 μmol CaHPO4/g diet.
ever, despite the observed decreases of luminal parameters and intestinal epitheliolysis on the PUFA-margarine diet, no significant reduction in proliferation was found with supplemental CaHPO₄ on this diet. Whether this lack of effect on colonic proliferation suggests an alternative or additional mechanism by which polyunsaturated fatty acids induce proliferation, e.g., by a direct effect on protein kinase C (47), is at present not known. Interestingly, the individual data points of rats fed CaHPO₄-supplemented diets lie on the same regression lines comparing cytolytic activity with epitheliolysis, and epitheliolysis with proliferation, as the points of rats fed control diets. In our opinion, this indicates that the main effect of a decreased proliferation caused by supplemental CaHPO₄ is due to the surfactant-dependent decreases in cytolytic activity and epitheliolysis. To our knowledge, this study with rats is the first one to demonstrate the sequence of effects of calcium on luminal and epithelial parameters. It should be stressed that our study does not prove cause-and-effect relationships, because it was done under steady-state conditions. However, because of the consistency with the in vitro and in vivo studies cited above, it is reasonable to speculate that this sequence of effects is causal.

Further information as to whether the sequence of luminal and epithelial effects of calcium observed in rats is also of relevance for studies of humans can be obtained by comparison of proliferation in colon biopsies with luminal parameters. For instance, preliminary results from our diet-controlled study with healthy volunteers indicate that supplemental calcium decreases soluble fatty acid concentrations and inhibits the cytolytic activity of fecal water (27), consistent with protective effects of dietary calcium on colonic proliferation (28, 29). Bruce et al. (48, 49) could not demonstrate a decrease in proliferation by supplemental calcium in placebo-controlled studies using patients with partial or subtotal colectomy. These operations may significantly alter bile acid metabolism. This will probably result in more primary bile acids that show less binding to calcium phosphate compared to secondary bile acids (18). Investigation of the solubility of bile acids and fatty acids and determination of luminal cytolytic activity in these studies would probably have provided more information about the nature of these results.

In conclusion, dietary CaHPO₄ supplementation of Western high-risk diets decreases the concentrations of soluble bile acids and fatty acids by precipitating these surfactants. Consequently, cytolytic activity of fecal water and intestinal epitheliolysis are decreased. These effects may explain the fat-type-dependent decreases in colonic cell proliferation after dietary supplementation with CaHPO₄. Ultimately, this sequence of effects may explain how diet could affect the risk of colon cancer.

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