Effects of dietary calcium and phosphate on the intestinal interactions between calcium, phosphate, fatty acids, and bile acids

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Abstract

Luminal free fatty acids and bile acids may damage the colonic epithelium and stimulate proliferation, which may increase the risk of colon cancer. It has been suggested that only soluble calcium ions (Ca^{2+}) precipitate fatty acids and bile acids, thus reducing their lytic activity. Consequently, precipitation of luminal Ca^{2+} by dietary phosphate should inhibit these effects. To evaluate the proposed antagonistic effects of dietary calcium and phosphate, we studied the intestinal interactions between calcium, phosphate, fatty acids, and bile acids in rats fed purified diets that differed only in the concentrations of calcium and phosphate. Increased dietary calcium drastically decreased the solubility of fatty acids in the ileum, colon, and faeces, as well as the solubility of bile acids in the colon and faeces. Although dietary calcium strongly increased the total faecal fatty acid concentration and hardly affected the total faecal bile acid concentration, the fatty acid and bile acid concentrations in faecal water were drastically decreased by dietary calcium. Consequently, the lytic activity of faecal water was decreased. Dietary phosphate did not interfere with these intestinal effects of calcium. These results indicate that dietary phosphate does not inhibit the protective effects of dietary calcium on luminal solubility and the lytic activity of fatty and bile acids.

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Epidemiological studies indicate that the incidence of colon cancer is positively correlated with the dietary intake of fat,¹⁻³ and negatively correlated with the intake of calcium.⁴ To explain these associations it has been hypothesised⁵ that dietary fat increases the colonic concentrations of soluble free fatty acids and secondary bile acids. These hydrophobic surfactants may damage colonic epithelial cells and consequently stimulate the proliferation of colonic crypt cells,^{6,7} which may increase the risk of colon cancer.⁸ With regard to the protective effects of dietary calcium, Newmark *et al*⁹ hypothesised that soluble calcium (Ca^{2+}) in the intestinal lumen precipitates fatty acids and bile acids and thus prevents their lytic effects on colonic epithelium. They suggested that dietary phosphate inhibits this protective effect of calcium, because phosphate will also bind luminal calcium ions and thus reduce the amount of calcium available for fatty acid and bile acid precipitation.

The protective effects of dietary calcium on the lytic activity of faecal water and on colonic

proliferation have been ascertained in many rodent and human studies.¹⁰⁻¹² However, the mechanism of these protective effects as well as the proposed inhibitory effect of phosphate have hardly been studied in vivo. Only Hu *et al*¹³ reported recently that phosphate inhibits the protective effects of calcium on epithelial proliferation caused by intrarectally instilled deoxycholate. Their study design, however, precluded the normal physiological interactions between dietary calcium, phosphate, fatty acids, and bile acids in the small and large intestine. In vitro experiments performed in our laboratory have shown that the lytic activity of bile acids increases in the presence of Ca^{2+} and decreases when phosphate is added. This inhibition is caused by the formation of insoluble precipitates of calcium, phosphate, and bile acid micelles.¹⁴ It should be noted that these in vitro results are contrast with the hypothesis mentioned above. Because phosphate is approximately equimolar to calcium in animal diets and is in molar excess of calcium in human diets, quantification of the intestinal interactions between calcium, phosphate, fatty acids, and bile acids in vivo is relevant for a proper evaluation of the effects of dietary calcium and phosphate. Therefore, in the present study these interactions were studied in a strictly controlled experiment with rats, using dietary calcium and phosphate concentrations which mimic their concentrations in animal and human diets. In addition, to determine the dietary effects on the cytolytic potency of the intestinal contents, luminal lytic activity was measured as lysis of erythrocytes by faecal water.

Methods

ANIMALS AND DIETS

Eight week old female outbred Wistar rats (Laboratory Animal Center of the Wageningen Agricultural University) were housed individually. For 2 weeks, eight groups of six rats each were fed purified diets, differing only in the concentrations of calcium and phosphate. The basal diet contained (per kg) 200 g casein (acid casein, DMV Veghel, The Netherlands), 471 g dextrose, 20 g cellulose, 35 g mineral mix, 10 g vitamin mix, 180 g palm oil, 20 g corn oil, and 10 g cholesterol. Cholesterol was added to increase the intestinal concentration of bile acids, because dietary cholesterol stimulates bile acid synthesis in rats.¹⁵ Because of the presence of casein, a phosphorylated protein, this basal diet contained 50 $\mu\text{mol/g}$ phosphate. Varying amounts of CaCO_3 and $\text{NaH}_2\text{PO}_4/\text{KH}_2\text{PO}_4$ (25, 75, and 225 $\mu\text{mol/g}$) were added to the basal diet

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in exchange for washed sand. These concentrations were chosen to cover a wide range around the recommended dietary concentration of 130 $\mu\text{mol/g}$ CaHPO_4 in the standard AIN-76 diet.¹⁶ The diets were balanced for sodium and potassium (final concentrations 125 and 100 $\mu\text{mol/g}$) with Na_2CO_3 and K_2CO_3 respectively. Dietary concentrations of other minerals and of vitamins were according to the AIN-76 recommendations.¹⁶

Diets and water were supplied *ad libitum*. Food consumption was recorded every 3 days and the animals were weighed weekly. Faeces were collected quantitatively from days 11 to 14.

The interactions between calcium, phosphate, fatty acids, and bile acids in the distal small intestine as well as in the colon were studied in detail in the groups with the lowest and the highest concentrations of dietary calcium and phosphate. Rats fed the low calcium (25 $\mu\text{mol/g}$) and phosphate (75 $\mu\text{mol/g}$) control diet or this diet supplemented with either 200 $\mu\text{mol/g}$ calcium or 200 $\mu\text{mol/g}$ calcium and phosphate were killed by decapitation on day 14 between 10.00 and 12.00 am. The distal third part of the small intestine (ileum) and the colon were excised and their contents collected.

TREATMENT OF ILEAL, COLONIC, AND FAECAL SAMPLES

Ileal contents were incubated in a shaking waterbath (5 minutes at 37°C) and subsequently centrifuged for 10 minutes at 15 000 *g* (Eppendorf 5415). Supernatant was stored at -20°C until further use and the pellet was freeze dried. Colonic contents and faeces were freeze dried. Colonic and faecal water were prepared by reconstituting freeze dried samples with double distilled water to respectively 20% and 35% dry weight, to mimic the conditions in the proximal and distal colon. After homogenisation, the samples were incubated in a shaking waterbath (1 hour at 37°C), followed by centrifugation for 10 minutes at 15 000 *g*. The supernatant was stored at -20°C until further use. Using this procedure, faecal water from freeze dried faeces in control experiments did not differ from faecal water prepared from fresh faeces with regard to concentrations of calcium, phosphate, fatty acids, and bile acids¹⁰ and with regard to lytic activity.¹⁵

ANALYSES

After dry ashing of the freeze dried samples, total calcium was measured using an atomic absorption spectrophotometer (Model 1100, Perkin-Elmer Corp, Norwalk, CT, USA) and total phosphate was measured as described by Fiske and Subbarow.¹⁷ Supernatants were acidified with trichloroacetic acid (final concentration 5% w/v) and analysed for calcium and phosphate as described above. Total and soluble fatty acids were determined as follows. Freeze dried samples and supernatants were acidified with HCl (final concentration 4 M) and fatty acids were subsequently extracted three times with 5 volumes of diethyl ether. After evaporation of diethyl ether, fatty acids were resolubilised in

ethanol and quantified using a colorimetric enzymatic assay (NEFA-C, Wako Chemicals, Neuss, Germany). In control experiments this extraction procedure resulted in >95% recovery of added fatty acids. Total bile acids were extracted from freeze dried samples with a *t*-butanol/water mixture (1:1, v/v), as described previously.¹⁸ Extracts and supernatants were assayed for bile acids using a fluorimetric enzymatic assay (Sterognost 3 α -flu, Nycomed AS, Oslo, Norway).

DETERMINATION OF LYTIC ACTIVITY

Human erythrocytes were isolated and washed as described by Coleman *et al.*¹⁹ Increasing volumes of faecal water (0–160 μl) were mixed with 154 mM NaCl to a total volume of 160 μl . After preincubation for 5 minutes at 37°C in a shaking waterbath, 40 μl erythrocytes (final haematocrit 5%) were added. Simultaneously, erythrocytes were incubated in 154 mM NaCl (0% lysis) and in double distilled water (100% lysis). After incubation for 30 minutes at 37°C, samples were centrifuged for a minute at 10 000 *g*. Haemolysis was determined by measuring the Fe content of the supernatant using an atomic absorption spectrophotometer (Perkin-Elmer 1100). The lytic activity was quantified as the area under the lytic curve and expressed as a percentage of the maximal area, which implies 100% lysis of erythrocytes at each dilution of faecal water. We recently found a high correlation between luminal lytic activity and *in vivo* colonic proliferation as determined by lysis of erythrocytes and ³H-thymidine incorporation, respectively.¹⁵

STATISTICS

Results are given as means of six rats per group with standard errors. After analysis of variance, differences between means were subjected to the least significant difference (LSD) test. Differences were regarded as significant if $p < 0.05$.

Results

No differences were observed between groups in food intake (mean 13.0 g/day) or in final body weight (mean 175 g). The daily output of faeces was slightly enhanced by dietary calcium and decreased by dietary phosphate (Table I).

First, we studied the intestinal interactions of calcium, phosphate, fatty acids, and bile acids in animals fed the low calcium and phosphate control diet or this diet supplemented with either calcium or calcium and phosphate. Figure 1 shows the effects of these diets on the concentra-

TABLE I Effects of diets on faecal output (g/day) (values, mean (SEM), $n=6$)

Dietary phosphate ($\mu\text{mol/g}$)	Dietary calcium ($\mu\text{mol/g}$)		
	25	75	225
75	1.19 (0.09) ^{A,a}	1.16 (0.05) ^A	1.60 (0.07) ^{B,a}
125	—	1.02 (0.10) ^A	1.60 (0.09) ^{B,a}
275	0.80 (0.03) ^{A,b}	1.02 (0.09) ^{AB}	1.21 (0.09) ^{B,b}

Values in the same row that do not share the same capital letter, and those in the same column that do not share the same letter are significantly different ($p < 0.05$).

Figure 1: Effects of the low calcium (25 $\mu\text{mol/g}$) and phosphate (75 $\mu\text{mol/g}$) control diet (open bars) and of the control diet supplemented with either 200 $\mu\text{mol/g}$ calcium (hatched bars) or 200 $\mu\text{mol/g}$ calcium and phosphate (filled bars) on the intestinal concentrations of soluble and insoluble calcium and phosphate. Whole bars reflect the total concentrations (mean (SEM), $n=6$). *Significant v the control diet ($p<0.05$). **Significant v the high calcium diet ($p<0.05$).

tions of soluble and insoluble calcium and phosphate in the ileum, colon, and faeces. High dietary calcium, as well as additional supplementation with phosphate, hardly affected the soluble concentrations of calcium and phosphate in the ileum, colon, and faeces. On the other hand, these treatments drastically increased the concentrations of insoluble calcium. Calcium supplementation increased the intestinal concentrations of insoluble phosphate, which indicates formation of insoluble calcium phosphate. This was stimulated further by additional supple-

TABLE II Effects of diets on the total concentrations ($\mu\text{mol/g}$) of fatty acids and bile acids in the ileum, colon, and faeces (values, mean (SEM), $n=6$)

Dietary calcium ($\mu\text{mol/g}$)	25	225	225
Dietary phosphate ($\mu\text{mol/g}$)	75	75	275
Ileum:			
Fatty acids	35 (4) ^a	150 (14) ^b	162 (19) ^b
Bile acids	80 (10)	56 (7)	68 (11)
Colon:			
Fatty acids	39 (3) ^a	495 (14) ^b	297 (35) ^c
Bile acids	19 (4)	16 (2)	18 (2)
Faeces:			
Fatty acids	97 (10) ^a	951 (29) ^b	704 (68) ^c
Bile acids	38 (5)	25 (2)	27 (3)

Values in the same row that do not share the same letter are significantly different ($p<0.05$).

Figure 2: Effects of the low calcium (25 $\mu\text{mol/g}$) and phosphate (75 $\mu\text{mol/g}$) control diet (open bars) and of the control diet supplemented with either 200 $\mu\text{mol/g}$ calcium (hatched bars) or 200 $\mu\text{mol/g}$ calcium and phosphate (filled bars) on the intestinal solubility of fatty acids and bile acids (mean (SEM), $n=6$). *Significant v the control diet ($p<0.05$). No significant effects of dietary phosphate were observed.

mentation with phosphate, especially in the colon and faeces.

The total fatty acid and bile acid concentrations along the intestine are shown in Table II. Because of these diet induced differences in total fatty acid concentrations, the solubility of fatty acids and bile acids is expressed relative to their total concentrations (Fig 2). Dietary calcium supplementation drastically reduced the solubility of fatty acids in the ileum, and this effect was maintained in the colon and faeces. Calcium supplementation did not influence the solubility of bile acids in the ileum, but it reduced their solubility to about 20% in the colon and faeces. Additional phosphate supplementation did not affect the calcium induced reduction in solubility of fatty acids and bile acids.

Because of the similarity of the effects observed in the colon and faeces, we subsequently studied the concentration dependence of the effects of dietary calcium and phosphate in the faeces. Figures 3 and 4 show the effects of the different diets on the total faecal concentrations of calcium, phosphate, fatty acids, and bile acids. As expected, increased dietary calcium increased the total calcium in faeces and this was only slightly stimulated by phosphate supplementation of the high calcium diet (Fig 3). Increased dietary calcium increased faecal phosphate because the formation of insoluble calcium phosphate inhibits the absorption of phosphate. This effect was more pronounced in rats taking the high phosphate diets. Dietary calcium supplementation drastically increased the total faecal fatty acid concentration (Fig 4) and this reflects the inhibition of the intestinal absorption of fatty acids. This inhibition was partly counteracted by increased dietary phosphate. In contrast to fatty acids, the total bile acid concentration was hardly influenced by the different diets. The observed slight decrease in faecal bile acid concentration with increasing dietary calcium concentrations reflects the concomitant increase in faecal mass (Table I). Therefore no significant

Figure 3: Effects of dietary calcium and phosphate ($\circ$ 75; $\bullet$ 125; $\bullet$ 275 $\mu\text{mol/g}$) on the total faecal concentrations of calcium and phosphate (mean (SEM), $n=6$). Different curves indicate a significant effect of dietary phosphate. SEMs are either smaller than symbols or indicated by bars.

Figure 4: Effects of dietary calcium and phosphate ($\circ$ 75; $\bullet$ 125; $\blacksquare$ 275 $\mu\text{mol/g}$) on the total faecal concentrations of fatty acids and bile acids (mean (SEM), $n=6$). Different curves indicate a significant effect of dietary phosphate. SEMs are either smaller than symbols or indicated by bars.

effect of the different diets on the total daily output of bile acids was observed.

We subsequently determined the *soluble* faecal concentrations of calcium, phosphate, fatty acids, and bile acids. Supplemental dietary calcium did not increase the concentrations of soluble calcium (Fig 5), notwithstanding the large increase in total faecal calcium. Only on the low calcium diet was soluble calcium significantly decreased, as soluble phosphate was increased by phosphate supplementation of this diet. Phosphate supplementation of the intermediate and high calcium diets increased the soluble phosphate value, but decreased the pH of faecal water. The net result of these antagonistic effects was that no significant change in soluble calcium was observed. The logarithm of the concentration product of calcium and phosphate was negatively correlated with the pH of faecal water ($r=-0.75$, $n=48$; $p<0.001$). This indicates that the soluble faecal concentrations of calcium and phosphate are controlled by the pH

Figure 5: Effects of dietary calcium and phosphate ($\circ$ 75; $\bullet$ 125; $\blacksquare$ 275 $\mu\text{mol/g}$) on the pH of faecal water on the soluble faecal concentrations of calcium and phosphate (mean (SEM), $n=6$). Different curves indicate a significant effect of dietary phosphate. SEMs are either smaller than symbols or indicated by bars.

dependent solubility product of calcium phosphate.¹⁰

Increased dietary calcium decreased the fatty acid concentration in faecal water (Fig 6). Supplementary dietary phosphate decreased the fatty acid concentration only on the low calcium diet. The bile acid concentration in faecal water also decreased with increasing dietary calcium, and this was not influenced by dietary phosphate. The logarithms of soluble fatty acid concentrations in the faeces were highly correlated with the logarithms of precipitated faecal calcium and phosphate concentrations (respectively -0.85 and -0.76 , $n=48$; $p<0.001$). Similar negative correlations were found for the logarithms of soluble bile acids and of precipitated calcium and phosphate (respectively -0.80 and -0.60 , $n=48$; $p<0.001$).

Finally, using lysis of erythrocytes, we ascertained whether the decreased luminal solubility of fatty acids and bile acids affected the cytolytic potency of the intestinal contents. The lytic activity of faecal water was high on the low calcium diets (Fig 6). Corresponding with the decreased solubility of fatty acids and bile acids, the lytic activity was completely inhibited by the highest level of dietary calcium and no significant effect of dietary phosphate was observed.

Discussion

These results show for the first time to our knowledge, the interactions between calcium, phosphate, fatty acids, and bile acids along the intestine. Extrapolating these results to human diets, the lowest calcium concentration used in the present study (25 $\mu\text{mol/g}$) reflects a daily intake of calcium of about 12.5 mmol (500 mg) which is about 60% of the recommended dietary allowance for calcium. Hence, increasing the calcium concentration to 75 and 225 $\mu\text{mol/g}$ mimics a supplementing the human diet with 25 and 100 mmol/day, respectively. The phosphate concentrations were changed accordingly to incorporate the molar calcium to phosphate ratio of about 0.5, which is typical for human diets.²⁰ It was shown that dietary phosphate does not interfere with the protective effects of dietary calcium on luminal solubility and lytic activity of fatty acids and bile acids. This is supported by our study with human volunteers,^{20,21} in which an excess of dietary phosphate did not prevent the protective effects of supplemental dietary calcium on solubility and lytic activity of luminal surfactants. Our study does not, therefore, offer an explanation for the recent observations of Hu *et al.*,¹³ who found that phosphate inhibits the protective effect of dietary calcium on colonic proliferation in rats. These authors, however, used intrarectal instillation of deoxycholate combined with short term (about 24 hours) oral intubations of calcium and phosphate. This design precludes, in our opinion, the normal physiological interaction between calcium, phosphate, and bile acids in the small and large intestine. Our results are in accordance with other studies in rodents, which showed that dietary supplementation with calcium phosphate inhibits lytic activity of faecal water and colonic proliferation.^{10,11,22-25} The extent of the reported

Figure 6: Effects of dietary calcium and phosphate (○ 75; ● 125; ● 275 $\mu\text{mol/g}$) on the soluble faecal concentrations of fatty acids and bile acids and on the lytic activity of faecal water (mean (SEM), $n=6$). Different curves indicate a significant effect of dietary phosphate. SEMs are either smaller than symbols or indicated by bars.

inhibition is comparable with that found in other studies using similar amounts of calcium carbonate or calcium lactate.^{7, 26–28} In our opinion, these results already suggest that the type of calcium salt does not interfere with the protective effects of dietary calcium on lytic activity of faecal water and on proliferation. In none of these studies, however, were the intestinal interactions between calcium, phosphate, fatty acids, and bile acids determined. Quantification of these interactions is essential for a proper discussion of the intestinal effects of dietary calcium and phosphate.

Dietary supplementation with calcium carbonate did not increase the soluble calcium concentrations along the intestine and in the faeces (Figs 1 and 5), notwithstanding the large increase in the total faecal calcium concentrations (Figs 1 and 3). The present results indicate that a substantial amount of this total calcium is associated with insoluble phosphate, which is in accordance with results obtained in other animal²⁹ and human²⁰ studies. As shown earlier,^{29, 30} calcium phosphate starts precipitating at a pH of about 6. Therefore, high concentrations of soluble calcium can only exist at the relatively low pH of the proximal small intestine,³¹ or when no phosphate is present, as can be derived from the observed negative correlation between the logarithm of the solubility product of calcium phosphate and the pH of faecal water.

Increased dietary calcium drastically increased the faecal excretion of fatty acids (Fig 4), which has also been found in other studies.^{28, 32} This reflects inhibition of the absorption of dietary fat. On the low phosphate diets this inhibition is probably caused by precipitation of fatty acids by high concentrations of soluble calcium in the proximal small intestine. As shown in Figure 2, this effect of calcium on the solubility of fatty

acids persisted in the ileum and colon. The stimulation of fatty acid excretion was slightly counteracted by dietary phosphate (Fig 4), which indicates that under these conditions, formation of insoluble calcium phosphate had already occurred in the proximal small intestine. Our results indicate that this effect of phosphate is confined to the proximal small intestine because increased dietary phosphate did not significantly increase the solubility of fatty acids in the ileum, colon, and faeces (Fig 2). We do not, at present, have an adequate explanation for the apparently anomalous effect of phosphate on the fatty acid concentration in faecal water in rats fed the low calcium diet, and this requires further investigation.

The present results show that dietary calcium has a site specific effect on the solubility of bile acids. It can be speculated that the calcium dependent precipitation of bile acids in the colon is a result of their microbial deconjugation and dehydroxylation in this compartment. Bile acids in the rat are predominantly conjugated with taurine.³³ As shown earlier,^{14, 30} taurine conjugated bile acids are not precipitated by calcium or calcium phosphate. In contrast, the unconjugated, carboxylic, bile acids are easily precipitated by calcium phosphate.³⁰

The observation that bile acids are not precipitated by calcium in the ileum, where reabsorption of bile acids takes place, explains why total faecal bile acid output is hardly affected by dietary calcium (Fig 4). In contrast, fatty acid excretion is strongly increased by dietary calcium, suggesting that fatty acids are precipitated by calcium before the absorption site, the proximal small intestine, is reached. These findings indicate that the total faecal concentrations of fatty acids and bile acids are not determined by the amount of intraluminally formed complexes, as was recently suggested by Appleton *et al.*,³⁴ but are determined by the site at which this occurs.

We found significant negative correlations in faeces between soluble fatty acids and bile acids and insoluble calcium and phosphate. Although correlations never prove a causal relationship, they may indicate that the solubilities of fatty acids and bile acids are determined by insoluble calcium phosphate. This is in accordance with our *in vitro* studies,^{14, 30} showing that calcium phosphate decreases the solubility of bile acids.

Finally, a pertinent finding of the present study is that the protective effect of dietary calcium on the lytic activity of faecal water correlated with its effect on the concentrations of soluble fatty acids and bile acids (Fig 6), but not with their total concentrations (Fig 4). In accordance with the results of several other studies,^{10, 14, 35} this indicates that these soluble surfactants are the main determinants of luminal lytic activity. We recently found that this luminal lytic activity is highly correlated with *in vivo* proliferation of colonic epithelium,^{11, 15} which indicates that these soluble surfactants may affect the risk of colon cancer.^{8, 36}

In conclusion these results show that dietary calcium stimulates the formation of insoluble calcium phosphate and decreases the luminal solubility of fatty acids and bile acids in rats. Luminal lytic activity is consequently inhibited.

These protective effects of calcium are not inhibited by a fourfold increase in dietary phosphate.

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