Red meat and colon cancer: dietary haem-induced colonic cytotoxicity and epithelial hyperproliferation are inhibited by calcium

Aloys L.A. Sesink, Denise S.M.L. Termont, Jan H. Kleibeuker and Roelof Van der Meer

Nutrition and Health Programme, Wageningen Centre for Food Sciences, NIZO Food Research, PO Box 20, 6710 BA Ede and 1Department of Gastroenterology, University Hospital, Groningen, The Netherlands

High intake of red meat is associated with increased colon cancer risk. We have shown earlier that this may be due to the high haem content of red meat, because dietary haem increased cytolytic activity of faecal water and colonic epithelial proliferation. Dietary calcium inhibits diet-induced epithelial hyperproliferation. Furthermore, it has been shown that supplemental calcium inhibited the recurrence of colorectal adenomas. Therefore, we studied whether dietary calcium phosphate can exert its protective effects by inhibiting the deleterious effects of haem. In vitro, calcium phosphate precipitated haem and inhibited the haem-induced cytotoxicity. Subsequently, rats were fed diets, differing in haem (0 or 1.3 µmol/g) and calcium phosphate content only (20 or 180 µmol/g). Faeces were collected for biochemical analyses. Cytolytic activity of faecal water was determined from the degree of lysis of erythrocytes by faecal water. Colonic epithelial proliferation was measured in vivo using [3H]thymidine incorporation. In rats fed low calcium diets, dietary haem increased cytoytic activity of faecal water (98 ± 1 versus 1 ± 1%, P < 0.001) and the concentration of cations in faeces (964 ± 31 versus 254 ± 20 µmol/g), when compared with controls. This indicates that dietary haem increased colonic mucosal exposure to luminal irritants. Colonic epithelial proliferation was increased compared with controls (70 ± 4 versus 48 ± 8 d.p.m./µg DNA, P < 0.001). This was accompanied by metabolism of the ingested haem and solubilization of haem compounds in the faecal water. A high calcium diet largely prevented this metabolism and solubilization. It also inhibited the haem-induced cytoytic activity of faecal water and increase in faecal cation concentration. In accordance, the haem-induced colonic epithelial hyperproliferation was prevented. We therefore suggest that dietary calcium phosphate acts as a chemopreventive agent in colon carcinogenesis by inhibiting the cytolytic and hyperproliferative effects of dietary haem.

Introduction

Colon cancer is one of the most prevalent cancers in western countries. The incidence of this multifactorial disease is strongly related to age, both in high- and low-risk countries (1). It is now generally accepted that a time-dependent clonal accumulation of multiple mutations in tumour suppressor genes and oncogenes results in the transformation of normal colonic epithelium into hyperproliferative tissue, adenoma, and finally, carcinoma (2). Accumulation of these mutations is favoured by a disturbance of the well-controlled epithelial cell turnover, determined by proliferation and cell death (2).

Many epidemiological studies indicate that a western-style diet is associated with a high colon cancer incidence. Especially, the consumption of red meat, and not of white meat, was positively associated with colon cancer (3,4). How dietary components like red meat influence colon cancer risk is not precisely known. Based on mutational analysis of colon cancers, Kinzler and Vogelstein argued that dietary factors that lead to colon cancer are probably not mutagens, but rather luminal irritants that damage epithelial cells (2). This leads to a compensatory epithelial regeneration, which increases the risk of endogenous mutations in cell-turnover genes. In line with this, we recently hypothesized that the association between red meat consumption and colon cancer might be due to the high haem content of red meat and not to meat-associated mutagens (5). In a rat study, we showed that dietary haem enhanced cytoytic activity of the faecal water, indicating increased exposure of the colonic mucosa to luminal irritants. In addition, colonic epithelial proliferation was increased, which is thought to reflect a higher risk for cancer (6,7). These effects were not mediated by well-known surfactants like bile acids or fatty acids, suggesting the involvement of an additional haem-induced cytotoxic factor. In accordance with our results, a recent study showed that in patients with a history of colonic adenomas, the labelling index in the upper part of the colonic crypt was increased among subjects with high red meat consumption (8).

Calcium is proposed to act as a chemopreventive agent in colon carcinogenesis (9). Intestinal calcium phosphate (CaPi) precipitates amphipathic compounds like bile acids and fatty acids and thus removes them from the faecal water (10). As a consequence, the cytoytic potential of the faecal water is lowered, which may result in a decreased colonic epithelial damage and proliferation. Evidence for these calcium-specific effects has already been given by several in vitro as well as in vivo studies (10–14). For instance, freshly formed calcium phosphate, but not ionic calcium, binds and precipitates unconjugated secondary bile acids and thus inhibits their cytoytic effect (15). CaPi also binds and precipitates the haem metabolite bilirubin in vitro (16) as well as in the intestinal lumen of rats (17) and humans (18). Because of the amphipathic similarity between bilirubin and haem, i.e. a hydrophobic tetrapyrrolic backbone with two polar side chains, CaPi might also precipitate native or modified haem molecules. We therefore hypothesise that increasing the CaPi content of a haem diet protects against the haem-induced cytotoxicity of the faecal water and colonic epithelial hyperproliferation. To test our hypothesis, we first determined in vitro whether CaPi could precipitate haem or the haem-induced cytotoxic factor. Secondly, in an in vivo study, rats were fed diets differing in

Abbreviations: CaPi, intestinal calcium phosphate; MOPS, 3-[N-morpholino]-propanesulfonic acid.

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Effects are inhibited by a high CaPi diet. The results of this study show that dietary CaPi largely inhibited the cytotoxic and hyperproliferative effects of haem on the rat colonic epithelium.

Materials and methods

In vitro experiments

We first studied whether freshly formed CaPi could bind and thus precipitate haem from a buffered aqueous solution. Haem (20 mM in 50 mM NaOH) was diluted in 100 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.0, to obtain a concentration range from 0 to 400 µM haem. CaPi was formed by mixing CaCl₂ and Na₂HPO₄ (final concentrations 10 mM) in 100 mM MOPS, pH 7.0 (final volume 100 µl). After incubating for 10 min at 37°C, the mixture was centrifuged for 2 min at 10 000 g. The supernatants were discarded and 100 µl haem solution (0–400 µM in 100 mM MOPS, pH 7.0) was added to the pellets. After vigorous mixing, the mixtures were incubated for 2 h at 37°C in the dark with frequent mixing and centrifuged at 10 000 g for 2 min. The remaining haem in solution was quantified, after a 20-fold dilution in double-distilled water, by measuring the absorbance of the supernatant at 400 nm with a Lambda 2 spectrophotometer (Perkin Elmer, Norwalk, CT, USA). We also studied if the haem-induced cytotoxic factor from faecal water was sensitive to precipitation by CaPi. For this, we used faecal water from rats from our previous study (cytolytic activity of the faecal water was stable during storage at –20°C), which were fed either a control diet or a haem-supplemented diet (5). In that study, we showed that addition of haem to the diet increased the cytotoxic activity of faecal water and the colonic epithelial proliferation, through the formation of a hitherto unknown cytolytic factor. This effect was highly specific for haem, because addition of other tetrapyrroles (protoporphyrin or bilirubin) or inorganic iron had no effect. To see if CaPi could inhibit this haem-induced cytotoxic activity, the experiment was performed exactly as described above, but now 100 µl faecal water was taken instead of 100 µl haem solution. Supernatants of the faecal water incubations were assayed for cytotoxicity as described below. These experiments were performed in triplicate.

Animals and diets

The experimental protocol was approved by the Animal Welfare Committee of the Agricultural University, Wageningen, The Netherlands. Nine-week-old male outbred Wistar rats (Harlan Horst/Wu, specific pathogen free; n = 32), mean body weight 243 g, were housed individually (n = 8 per diet) in metabolic cages in a room with controlled temperature (22–24°C), relative humidity (50–60%) and light/dark cycle (lights on from 6 a.m. to 6 p.m.). During 2 weeks, rats were fed purified diets, differing only in CaPi and haem content. The low-calcium diets contained, per kg 200 g casein, 528 g dextrose, 204 g corn oil, 7% (w/v) sodium taurocholate and 20 mmol calcium carbonate (CaHPO₄·2H₂O; Fluka, Zwijndrecht, The Netherlands). The fatty acid composition of the blend of palm oil and corn oil mimics the ratio of saturated to monounsaturated to polyunsaturated fatty acids (44:38:18) in a human Western diet. High-calcium diets contained 180 mmol CaHPO₄·2H₂O per kg, and this was exchanged for dextrose. For both levels of calcium, the diet was supplemented with 1.3 mmol haem per kg diet. To maintain equimolar ion concentrations in all diets, 1.3 mmol ferric citrate was added per kg control diet. Other minerals and vitamins, including choline (as choline chloride), were added according to Reeves et al. (19). Two additional groups of rats were fed the low-calcium diets to provide samples of the ileal contents free of possible [methyl-H]thymidine contamination. Food intake and body weights were recorded every 2–4 days. Faeces were collected quantitatively during days 11–14 of the experiment and frozen immediately at –20°C.

In vivo epithelial proliferation

After the experimental feeding period of 14 days, colonic and ileal scrapings were prepared and DNA and protein content of the scrapings were determined as described previously (5). The proliferative activity of the colonic and ileal epithelium was quantified by the incorporation of [methyl-H]thymidine (Amersham, Little Chalfont, UK) per µg DNA (5). This method has been shown to correlate highly with the crypt cell production rate per hour (20). We chose this quantitative, biochemical method because the main focus of our work is to study dietary modulation of luminal risk factors for colorectal cancer and their impact on proliferation in total intestinal epithelium. Moreover, the validity of the technically more complicated immunohistochemical measurements of proliferation in colon seems to be uncertain, as discussed recently (21,22).

Faecal water preparation

Faeces were freeze-dried after collection. Faecal water was prepared by reconstituting the freeze-dried faeces with appropriate amounts of double-distilled water as described previously (5).

Preparation of ileal water

For rats fed the low-calcium diets, which were not used in the proliferation experiments, the contents of the middle third of the small intestine (proximal ileum) were collected. The water phase of the content of the small intestinal lumen was obtained by centrifuging for 2 min at 14 000 g. The remaining supernatants (ileal water) were stored at –20°C until analysis.

Cytolytic activity of faecal and ileal water

This was determined by mixing 10 µl faecal water or 10 µl ileum water (the latter was first diluted 10-fold with saline) with 70 µl saline and incubating for 5 min at 37°C. Then, 20 µl of a washed human erythrocyte suspension were added (final hematocrit in assay 5%) and incubated for 15 min at 37°C. Subsequently, cytotoxicity was quantified as described previously (23). The relevance of this bioassay with erythrocytes for effects on intestinal epithelial cells was given by the high correlation coefficient (r = 0.97) between the lytic effects of mixtures of bile acids and fatty acids on human erythrocytes and on the human colon carcinoma-derived Caco-2 cells (24).

Analyses of faeces and faecal water

Sodium, potassium and ammonium in total faeces were determined as described previously (5). Percentage water of faeces for each group was calculated with the assumption that the total amount of sodium, potassium and ammonium and their counterions provided an osmolarity of 300 mosmol/l in faeces (25). For spectrophotometric analyses, an acidified chloroform–methanol extract (1 M HCl final concentration) of the faecal waters was obtained (26). The chloroform phase was dried under nitrogen and resolubilized in methanol. An absorption spectrum was recorded from 300 to 700 nm on a Lambda 2 spectrophotometer (Perkin-Elmer). Calcium and inorganic phosphate were determined as described previously (27). The amount of haem in faecal water and in total faeces was determined by a modified HemoQuant assay (28), using haemin as standard. For the measurement of haem in faecal water, 50 µl faecal water was diluted in 250 µl 5:1 (v/v) 2-propanol/1 M HCl. After mixing, samples were centrifuged for 2 min at 10 000 g. Subsequently, the haem concentration in the supernatant was measured as described (28). To quantify haem in whole faeces, an acidified methanol–chloroform extract (1 M HCl final concentration) (26) was obtained from ~20 mg faeces. The chloroform phase of the samples was dried under nitrogen and solubilized in 0.45 ml 250 mM KOH, followed by sonication for 5 min (Sonorex HR255 sonicating waterbath, Bandelin). When samples were properly dissolved, 0.45 ml double-distilled water, 3.75 ml 2-propanol and 0.75 ml 1.15 M HCl were added. After mixing, the samples were centrifuged for 10 min at 1500 g and the supernatants were assayed for their haem content as described (28). Fluorescence of the standards, samples and blanks was measured using excitation and emission wavelengths of 409 and 605 nm, respectively (LS 50B; Perkin-Elmer). Using these procedures, recovery of haem and protoporphyrin was respectively 92 ± 3 and 95 ± 3% for faecal water and 99 ± 6 and 83 ± 1% for whole faeces.

Statistics

Results are presented as mean ± SEM (n = 8). Statistics were calculated with a commercially available package (SPSS/PC, ver. 2.0; SPSS, Chicago, IL). To see if the observed haem effects were dependent on the level of dietary calcium, two-way analysis of variance was performed. Then we tested differences between each haem group and its calcium-matched control group, between the two control groups and between the two haem groups. Distribution of data was evaluated using normal probability plots. Equality of variances between groups was tested using the Levene test. In case of normally distributed data, the Student’s t-test (equal variances) or the Student’s t-test with Welch estimate (unequal variances) was used to test for differences between means (two-sided). When data were not normally distributed, differences between means were tested with the Mann–Whitney U-test (two-sided). In all cases, Bonferroni correction was made for the number of equations (n = 4).

Results

In vitro experiments

Analagous to our earlier work on bile acids (29) and on bilirubin (16), we explored whether haem and the haem-induced cytolytic factor were sensitive to precipitation by freshly formed amorphous CaPi. When buffered solutions containing 0–400 µM haem (pH 7.0) were co-incubated with 10 mM CaPi, haem was
freshly formed CaPi. Results are given as mean of three separate experiments (the SEMs are smaller than the size of the symbols).

Fig. 1. Effect of CaPi on the solubility of haem in vitro. Haem (0–400 µM) was incubated in buffer (pH 7.0) in the absence (○) or presence (●) of 10 mM freshly formed CaPi. Results are given as mean of three separate experiments.

Fig. 2. Effect of freshly formed CaPi on cytotoxicity of faecal water in vitro. Faecal water of control rats and of haem-fed rats was incubated in the absence (filled bars) or presence (hatched bars) of 10 mM CaPi. Bars show the mean ± SEM of three separate experiments.

completely precipitated (Figure 1). When faecal water, obtained from an earlier study with haem-fed rats (5), was pre-incubated with freshly prepared 10 mM CaPi for 2 h and subsequently centrifuged, cytotoxicity in the remaining supernatant drastically decreased, when compared with samples devoid of CaPi (Figure 2).

Animals, diets and effects on faecal characteristics

These in vitro results prompted us to investigate whether CaPi also attenuates the deleterious effects of haem in vivo. Therefore, rats were fed diets that differed only in haem and CaPi content. Food intake did not depend on the presence of haem in the diet, but was slightly higher on both high-calcium diets (Table I). During the experiment, a small, non-significant reduction of growth was seen in the haem-fed rats. Daily faecal output of dry matter was not affected by haem on either the low-calcium or the high-calcium diet, but was markedly increased by both high-calcium diets, in line with earlier studies (27). As we observed softening of the faeces in the low-calcium haem group, we quantified the hydration of the faeces by measuring the total amount of sodium, potassium and ammonium. On the low-calcium diet, supplemental haem drastically increased the concentration of total faecal cations, and consequently, the percentage weight of the faeces. This was largely due to the 10-fold increase in faecal sodium content. Potassium and ammonium content of the faeces were raised 3- and 2-fold, respectively, by dietary haem. These haem-induced increases in faecal cations and faecal wet weight were counteracted by addition of calcium phosphate to the diet, though not completely.

We have suggested previously that the deleterious effects of haem might be mediated by luminal degradation or conversion of the ingested haem (5). Table II shows that the low- and the high-calcium haem group had a comparable daily intake of haem. Daily faecal output of haem was low in both control groups. When the diet was supplemented with haem, excretion of haem rose substantially on both calcium levels, and this increase was larger in the high-calcium group. Consequently, less haem was metabolized during gastrointestinal transit in rats on a high-calcium diet.

When preparing the faecal waters, we noted a large difference in colour. The faecal water of the low-calcium haem group was dark-brown, whereas the faecal waters of the other groups, including the high-calcium haem group, had a light-brown appearance. We quantified this by recording the absorption

| Table I. Effect of dietary CaPi and haem on daily food intake, animal growth and faecal characteristics |
|---------------------------------|-----------------|-----------------|
|                                 | Low-calcium diet | High-calcium diet |
|                                 | Control | Haem        | Control | Haem        |
| Food intake (g/day)             | 17.0 ± 0.3 | 16.4 ± 0.4   | 18.1 ± 0.5 | 17.7 ± 0.4   |
| Growth (g/day)                  | 3.5 ± 0.3    | 2.9 ± 0.3    | 3.7 ± 0.3   | 3.0 ± 0.3    |
| Faecal output (dry) (g/day)     | 0.61 ± 0.02a | 0.69 ± 0.04a | 1.55 ± 0.07b | 1.64 ± 0.06b |
| Faecal output haem (µmol/g)     | 254 ± 20a    | 964 ± 31b    | 192 ± 26a   | 313 ± 18b    |
| Sodium (µmol/day)               | 50 ± 6a      | 487 ± 21b    | 34 ± 5a     | 125 ± 13b    |
| Potassium (µmol/day)            | 82 ± 8b      | 267 ± 7b     | 33 ± 6c     | 67 ± 6d      |
| Ammonium (µmol/day)             | 122 ± 8b     | 211 ± 18b    | 125 ± 20a   | 121 ± 6a     |
| Wet weight (%)                  | 62 ± 2a      | 86 ± 1b      | 54 ± 3b     | 67 ± 1c      |

Percentage water of faeces for each group separately was calculated with the assumption that the total amount of sodium, potassium and ammonium and their counterions provided an osmolarity of 300 mosmol/l in faeces. Values represent means ± SEM (n = 8). Values in the same row not sharing the same superscript are significantly different (P < 0.05).

| Table II. Effect of dietary CaPi and haem on haem intake and on faecal water parameters |
|---------------------------------|-----------------|-----------------|
|                                 | Low-calcium diet | High-calcium diet |
|                                 | Control | Haem        | Control | Haem        |
| Intake haem (µmol/day)          | 0       | 21.4 ± 0.5  | 0       | 23.0 ± 0.5  |
| Faecal output haem (µmol/day)   | 0.2 ± 0.08   | 11.0 ± 0.7b  | 0.4 ± 0.08 | 18.5 ± 0.8d |
| Metabolized haem (µmol/day)     | 0       | 10.4 ± 0.2a | 0       | 4.5 ± 0.6b  |
| Haem in faecal water (µM)       | 6 ± 1a   | 35 ± 5b     | 0 ± 1c   | 5 ± 0d      |
| Ca in faecal water (µM)         | 0.79 ± 0.12a | 0.53 ± 0.10a | 2.62 ± 0.31b | 2.82 ± 0.13b |
| Phosphate in faecal water (mM)  | 1.2 ± 0.2a | 3.7 ± 0.2b  | 7.7 ± 1.0c | 7.7 ± 0.8c  |

Haem intake was calculated by multiplying daily food intake by the haem content of the diet. The amount of metabolized haem was calculated by subtracting faecal haem output from daily intake of haem. Values are given as means ± SEM (n = 8). Values in the same row not sharing the same superscript are significantly different (P < 0.05).
In contrast to the effects in faecal water, haem did not significantly affect cytotoxicity of ileal contents (Table II). Addition of haem increased the amount of haem in faecal water on the low-calcium diet, but only minor amounts were solubilized on the high-calcium diet. Supplemental calcium, but not haem, increased the concentration of calcium in faecal water. Addition of haem increased the concentration of inorganic phosphate on the low-calcium diet, but not on the high-calcium diet. It should be noted that the concentration of calcium and phosphate in faecal water is only a very small fraction of their total faecal concentrations. In accordance with our earlier study (27), we found that dietary calcium phosphate increases faecal calcium from 122 ± 5 to 1659 ± 27 μmol/g and faecal phosphate from 47 ± 3 to 788 ± 23 μmol/g, with no significant effect of haem.

Subsequently, we tested whether the solubilization of haem in the faecal water and the inhibition by dietary calcium corresponded to changes in cytolytic activity of the faecal water. No cytolytic activity was observed in faecal waters of either control group (Figure 4). Faecal water of rats fed the low-calcium haem diet was highly cytotoxic. In contrast, no cytolytic activity was observed in faecal waters of the high-calcium haem group.

**Effects of haem and calcium on colonic epithelial proliferation**

The effect of the diet on the proliferative activity of the colonic epithelial cells was similar to that on the cytotoxicity of the faecal water (Figure 5). There was no difference in proliferative activity between low- and high-calcium control groups. Dietary haem significantly increased proliferation in the low-calcium group, but this effect was absent in the high-calcium diet. Furthermore, there was no dietary effect on either the mucosal DNA content (1107 ± 50 μg/scraping) or the protein content (9.6 ± 2.1 mg/scraping).

**Effects of diets on ileum**

In contrast to the effects in faecal water, haem did not significantly affect cytotoxicity of ileal contents on a low-calcium diet (90 ± 3 versus 77 ± 8% for haem and control group, respectively). In addition, dietary haem did not enhance proliferation of the epithelial cells of the ileum in either a low or a high-calcium background (in d.p.m./μg DNA: low-calcium control, 45 ± 7; low-calcium haem, 39 ± 5; high-calcium control, 45 ± 5; high-calcium haem, 40 ± 6).

**Discussion**

To our knowledge, an interaction between CaPi and haem concerning the solubility and metabolism of the latter has not been documented before. Our in vitro experiments showed that freshly formed CaPi, binds to and precipitates the native haem molecule. In addition, CaPi diminished cytotoxicity of faecal water of haem-fed rats, indicating that CaPi also bound the haem-induced cytotoxic factor. In vivo, dietary calcium also inhibited haem-induced cytotoxicity of the faecal water, and consequently, prevented the hyperproliferation of the colonic mucosa. Our results show that this cytoprotective effect is not related to soluble calcium, but is related to the concentration of insoluble calcium phosphate in faeces. This mechanism may contribute to the observed protective effect of calcium on colon cancer risk, because increased proliferation is commonly regarded as a risk factor in carcinogenesis (6,30).

We have shown previously that bile acids, fatty acids or haem itself were not responsible for the deleterious effects of dietary haem (5). Instead, we suggested that an unknown cytolytic factor was formed during the gastrointestinal passage of haem. Table

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Fig. 3. UV–visible absorbance spectrum of a lipid extract of pooled faecal waters of the different dietary groups. (----) low-calcium control; (-----) low-calcium haem; (···) high-calcium control; (-----) high-calcium haem.

Fig. 4. Effects of dietary haem and CaPi on the cytolytic activity of 20 μl faecal water. Results are shown as mean ± SEM. Bars, faecal water of control rats. Filled bars, faecal water of haem-fed rats. An asterisk indicates a statistically significant difference between haem-fed rats and their calcium-matched controls (P < 0.001).

Fig. 5. Effects of dietary haem and CaPi on the colonic epithelial proliferation (d.p.m. /μg DNA). Results are shown as mean ± SEM. Hatched bars, control rats. Filled bars, haem-fed rats. An asterisk indicates a statistically significant difference between haem-fed rats and their calcium-matched controls (P < 0.05).
Red meat and colon cancer

II shows that both the low- and high-calcium haem groups consumed the same amount of haem. Recovery of dietary haem in faeces of rats consuming the low-calcium diet was ~53% of the intake, whereas recovery was 83% in rats fed the high-calcium diet. Because total absorption of iron in the small intestine is ~10% of the daily intake (5) our data indicate that on the low-calcium diet substantial intestinal haem metabolism must have taken place. Increasing the calcium content of the diet and the subsequent precipitation of haem in the intestinal lumen, analogous to the observed \textit{in vitro} effects (Figure 1), may have resulted in a decrease of the dietary haem available for metabolism. Interestingly, consumption of the high-calcium diet also diminished the solubilization of haem compounds in the faecal water and protected against the haem-induced cytotoxicity of the faecal water. This suggests that these events are closely related, and therefore it can be speculated that the cytotoxic factor is formed during the metabolic conversion of haem. The structural elucidation of the haem-induced cytotoxic factor might reveal the underlying mechanism, and this will be the subject of future research. Preliminary results suggest that the haem-induced cytolytic activity of faecal water is caused by covalently modified haem metabolites.

The effect of dietary calcium on faecal recovery of haem is noteworthy and may limit the diagnostic value of the HemQuant method for the detection of intestinal bleeding. Our results indicate that the large variance in faecal recovery of dietary haem in humans (31) may partly be due to differences in calcium intake.

Our bioassay for cytolytic activity measures the sensitivity of erythrocyte membrane to lytic compounds in the faecal water. We have shown earlier that lytic effects of surfactants on erythrocytes and on Caco-2 cells were very similar (24) and that the cytolytic activity of faecal water and epithelial damage were highly correlated (27). Therefore, our bioassay reflects the exposure of the colonic epithelium to luminal irritants. The inhibition of the haem-induced cytolytic activity of faecal water by the high-calcium diet in our bioassay thus implies that under high-calcium conditions, the colonic mucosa is less exposed to cytolytic agents from the faecal stream, resulting in less epithelial damage. This is substantiated by the observation that dietary CaPi, which has been reported to date. But our earlier studies showed that the intestinal interaction between calcium, phosphate and cytoxic surfactants (such as bile acids, fatty acids and bilirubin, which is a haem metabolite) is similar in rats and humans (10,18). It is therefore reasonable to assume that CaPi can also bind haem or haem-metabolites in the human intestine. Providing that the results from the present study can be extrapolated to humans, this suggests that a relationship between red meat consumption and colon cancer can only be found in populations with a relatively low calcium intake. This may explain, at least partly, why the association between red meat and colon cancer is more frequently observed in epidemiological studies from the US than from Western Europe, because calcium intake is higher in the latter countries (13,38–41). With regard to the protective effect of dietary calcium in humans, it has been shown before that calcium, either given as a calcium carbonate supplement (27) or as a natural component in dairy products (12,23), decreased cytotoxicity of faecal water. In addition, several studies (11,13,14), but not all (42–44), showed an inhibitory effect of supplemental or dietary calcium on colonic epithelial proliferation or showed a normalization of the distribution of proliferating cells in the colonic crypt. Of course, hyperproliferation is only an early event, so therefore these studies cannot give a definite answer whether a high-calcium intake is protective in colon carcinogenesis. Recently, the recurrence of colonic adenomas was used as a primary endpoint in several human studies. High dietary calcium intake (45) or calcium supplements (46) were associated with a lower risk of recurrent adenomas. Stronger evidence comes from the calcium-intervention study of Baron \textit{et al.} (40), in which supplemental calcium decreased adenoma recurrence by ~25%. In addition, in the European intervention study, a modest, though not statistically significant, preventive effect of calcium was reported (47). In the study of Baron \textit{et al.} (40), however, supplemental calcium did not inhibit the epithelial proliferation effect in colonic biopsies (48), indicating that the predictive value of epithelial proliferation in biopsies is low. We feel that this is partly due to the limited reproducibility and validity of this method (21,22). Epithelial hyperproliferation \textit{in vivo} certainly is a risk factor for carcinogenesis due to its

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<tr>
<th>Diet-dependent effects</th>
<th>Response of colonic epithelium</th>
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<tbody>
<tr>
<td>Nutrient</td>
<td>Colonic lumen</td>
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<tr>
<td>Red meat</td>
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Fig. 6. Proposed mechanism for the interaction between dietary calcium phosphate and haem in the colonic lumen and its effect on the colonic epithelium.

haem are only observed at a low dietary calcium content. Our low-calcium control diet contained 20 \(\mu\)mol calcium/g dry weight mimicking a human daily intake of 400 mg (10 mmol) of calcium and a daily dry weight intake of 500 g food.

Because our diets mimicked the composition of human western-style diets, our results may have implications for the human situation. In the small intestine, haem is released from the meat proteins haemoglobin and myoglobin by the action of digestive enzymes (37). Furthermore, it has been shown that red meat consumption substantially increases faecal haem content (31). However, no studies concerning the effects of haem on cytolytic activity of faecal water and proliferation in the human colon have been reported to date. But our earlier studies showed that the intestinal interaction between calcium, phosphate and cytoxic surfactants (such as bile acids, fatty acids and bilirubin, which is a haem metabolite) is similar in rats and humans (10,18). It is therefore reasonable to assume that CaPi can also bind haem or haem-metabolites in the human intestine. Providing that the results from the present study can be extrapolated to humans, this suggests that a relationship between red meat consumption and colon cancer can only be found in populations with a relatively low calcium intake. This may explain, at least partly, why the association between red meat and colon cancer is more frequently observed in epidemiological studies from the US than from Western Europe, because calcium intake is higher in the latter countries (13,38–41). With regard to the protective effect of dietary calcium in humans, it has been shown before that calcium, either given as a calcium carbonate supplement (27) or as a natural component in dairy products (12,23), decreased cytotoxicity of faecal water. In addition, several studies (11,13,14), but not all (42–44), showed an inhibitory effect of supplemental or dietary calcium on colonic epithelial proliferation or showed a normalization of the distribution of proliferating cells in the colonic crypt. Of course, hyperproliferation is only an early event, so therefore these studies cannot give a definite answer whether a high-calcium intake is protective in colon carcinogenesis. Recently, the recurrence of colonic adenomas was used as a primary endpoint in several human studies. High dietary calcium intake (45) or calcium supplements (46) were associated with a lower risk of recurrent adenomas. Stronger evidence comes from the calcium-intervention study of Baron \textit{et al.} (40), in which supplemental calcium decreased adenoma recurrence by ~25%. In addition, in the European intervention study, a modest, though not statistically significant, preventive effect of calcium was reported (47). In the study of Baron \textit{et al.} (40), however, supplemental calcium did not inhibit the epithelial proliferation effect in colonic biopsies (48), indicating that the predictive value of epithelial proliferation in biopsies is low. We feel that this is partly due to the limited reproducibility and validity of this method (21,22). Epithelial hyperproliferation \textit{in vivo} certainly is a risk factor for carcinogenesis due to its
intrinsic property to increase random mutation rate above its natural background (7,30). Therefore, we think that the protective effect of CaPi on the haem-induced colonic cytotoxicity and epithelial hyperproliferation in vivo, as shown in this study, is relevant for the human situation.

To summarize, our hypothesis concerning the protective effect of dietary calcium on colon carcinogenesis is depicted in Figure 6. A high CaPi diet precipitates dietary haem and thus prevents the formation and solubilization of the haem-induced cytolytic factor in the faecal water. Consequently, the haem-induced increase in cytolytic activity of faecal water is inhibited. We showed that this is accompanied by an antiproliferative effect on the colonic epithelium, which may inhibit the carcinogenic process. Thus, by diminishing the detrimental effects of haem on the colonic mucosa, dietary calcium phosphate may contribute to the protection against colon cancer.

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Red meat and colon cancer


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