Red meat and colon cancer: dietary heme-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

Submitted for publication
ABSTRACT

High intake of red meat is associated with increased colon cancer risk. We have shown earlier that this may be due to the high heme content of red meat, because dietary heme increased cytolytic activity of fecal 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 heme. In vitro, calcium phosphate precipitated heme and inhibited the heme-induced cytotoxicity. Subsequently, rats were fed diets, differing in heme (0 or 1.3 µmol/g) and calcium phosphate content only (20 or 180 µmol/g). Feces were collected for biochemical analyses. Cytolytic activity of fecal water was determined from the degree of lysis of erythrocytes by fecal water. Colonic epithelial proliferation was measured in vivo using [3H]-thymidine incorporation. In rats fed low calcium diets, dietary heme increased cytolytic activity of fecal water (98 ± 1% versus 1 ± 1%, P<0.001) and the concentration of cations in feces (964 ± 31 versus 254 ± 20 µmol/g), when compared to controls. This indicates that dietary heme increased colonic mucosal exposure to luminal irritants. Colonic epithelial proliferation was increased compared to controls (70 ± 4 versus 48 ± 8 dpm/µg DNA, P<0.001). This was accompanied by metabolism of the ingested heme and solubilization of heme compounds in the fecal water. A high calcium diet largely prevented this metabolism and solubilization. It also inhibited the heme-induced cytolytic activity of fecal water and increase in fecal cation concentration. In accordance, the heme-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 heme.

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 heme content of red meat and not to meat-associated mutagens (5). In a rat study, we showed that dietary heme enhanced cytolytic activity of the fecal 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 heme-induced cytotoxic factor. In accordance with our results, a recent study showed that in patients with a history of colonic adenomas, the labeling 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 fecal water (10). As a consequence, the cytolytic potential of the fecal 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). CaPi also binds and precipitates the heme metabolite bilirubin in the intestinal lumen of humans (15). Because of the amphipathic similarity between bilirubin and heme, i.e. a hydrophobic tetrapyrolic backbone with two polar side chains, CaPi might also precipitate native or modified heme molecules. We therefore hypothesize that increasing the CaPi content of a heme diet protects against the heme-induced cytotoxicity of the fecal water and colonic epithelial hyperproliferation. To test our hypothesis, we first determined in vitro whether CaPi could bind and thus precipitate heme from a buffered aqueous solution. Heme (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 heme. 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 minutes at 37°C, the mixture was centrifuged for 2 minutes at 10,000g. The supernatants were discarded and 100 µL heme 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 hours at 37 °C in the dark with

Materials and methods

**In vitro experiments**

We first studied whether freshly formed CaPi could bind and thus precipitate heme from a buffered aqueous solution. Heme (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 heme. 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 minutes at 37°C, the mixture was centrifuged for 2 minutes at 10,000g. The supernatants were discarded and 100 µL heme 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 hours at 37 °C in the dark with
frequent mixing, and centrifuged at 10,000g for 2 minutes. The remaining heme in solution was quantified, after a 20-fold dilution in double-distilled water, by measuring the absorbance at 400 nm of the supernatant (Perkin Elmer, Lambda 2, Norwalk, MO, US). We also studied if the heme-induced cytotoxic factor from fecal water was sensitive to precipitation by CaPi. For this, we used fecal water from rats from our previous study (cytolytic activity of the fecal water was stable during storage at $-20^\circ\text{C}$), which were fed either a control diet or a heme-supplemented diet (5). In that study, we showed that addition of heme to the diet increased the cytolytic activity of fecal water and the colonic epithelial proliferation, through the formation of a hitherto unknown cytolytic factor. This effect was highly specific for heme, because addition of other tetrapyrroles (protoporphyrin or bilirubin) or inorganic iron had no effect. To see if CaPi could inhibit this heme-induced cytolytic activity, the experiment was performed exactly as described above, but now 100 µL of fecal water was taken instead of 100 µL of the heme solution. Supernatants of the fecal 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 243g, 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 AM to 6 PM). During two weeks, rats were fed purified diets, differing only in CaPi and heme content. The low-calcium diets contained, per kg 200 g casein, 528 g dextrose, 204 g fat (consisting of a blend of 82% palm oil and 18% corn oil), 20 g cellulose and 20 mmol calcium phosphate (CaHPO$_4 \cdot$2H$_2$O, Fluka). The fatty acid composition of the blend of palm oil and corn oil mimics the ratio of saturated to monounsaturated to polyunsaturated fatty acids (44:38:18) in a human Western diet. High-calcium diets contained 180 mmol CaHPO$_4 \cdot$2H$_2$O per kg, and this was exchanged for dextrose. For both levels of calcium, the diet was supplemented with 1.3 mmol heme per kg diet. To maintain equimolar iron concentrations in all diets, 1.3 mmol ferric citrate was added per kg control diet. Other minerals and vitamins, including choline (as choline chloride), were added according to the American Institute of Nutrition 1993 recommendations (16). Two additional groups of rats were fed the low-calcium diets to provide samples of the ileal contents free of [methyl-$^3$H]thymidine (see “In vivo colonic proliferation” below). Food intake and body weights were recorded every 2-4 days. Feces were collected quantitatively during days 11-14 of the experiment and frozen immediately at -20°C.

**In vivo colonic epithelial proliferation**

After the experimental feeding period of 14 days, colonic scrapings were prepared and DNA and protein content of the colonic scrapings were determined as described before (5). The proliferative activity of the colonic epithelium was quantified by the incorporation of [methyl-$^3$H]thymidine.
3H]thymidine (Amersham International, Amersham, UK) per µg DNA (5). This method has been shown to correlate highly with the crypt cell production rate per hour (17). We chose this quantitative, biochemical method because the main focus of our work is to study dietary modulation of luminal risk factors for colon cancer and their impact on proliferation in total colonic epithelium. Moreover, the validity of the technically more complicated immunohistochemical measurements of proliferation in colon seems to be uncertain, as discussed recently (18, 19).

**Fecal water preparation**

Feces were freeze-dried after collection. Fecal water was prepared by reconstituting the freeze-dried feces 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 was obtained by centrifuging for 2 minutes at 14000g. The remaining supernatants (ileal water) were stored at -20°C until analysis.

**Cytolytic activity of fecal and ileal water**

This was determined by mixing 10 µL of fecal water or 10 µL of ileum water (the latter was first diluted tenfold with saline) with 70 µL saline and incubating for 5 minutes at 37°C. Then, 20 µL of a washed human erythrocyte suspension were added (final hematocrit in assay 5%) and incubated for 15 minutes at 37°C. Subsequently, cytotoxicity was quantified as described previously (20). 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 (21).

**Analyses of feces and fecal water**

Sodium, potassium, and ammonium in total feces were determined as described previously (5). Percentage water of feces 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 feces (22). For spectrophotometric analyses, an acidified chloroform-methanol extract (final HCl-concentration, 1M) of the fecal waters was obtained (23). The chloroform phase was dried under nitrogen and resolubilized in methanol. An absorption spectrum was recorded from 300 to 700 nm on a spectrophotometer (Perkin-Elmer, Lambda 2, Norwalk, MO). The amount of heme in fecal water and in total feces was determined by a modified HemoQuant assay (24), using hemin as standard. For the measurement of heme in fecal water, 50 µL of fecal water was diluted in 250 µL of a 5:1 (v/v)
mixture of 2-propanol and 1M HCl. After mixing, samples were centrifuged for 2 minutes at 10000g. Subsequently, the heme concentration in the supernatant was measured as described (24). To quantify heme in whole feces, an acidified methanol-chloroform extract (final HCl concentration 1M) (23) was obtained from approximately 20 mg of feces. The chloroform phase of the samples was dried under nitrogen and solubilised in 0.45 mL 250 mM KOH, followed by sonication for 5 minutes (Sonorex RH255 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 minutes at 1500g and the supernatants were assayed for their heme content as described (24). Fluorescence of the standards, samples, and blanks was measured, using excitation and emission wavelengths of 409 and 605 nm, respectively (Perkin Elmer, LS 50B, Norwalk, MO). Using these procedures, recovery of heme and protoporphyrin was respectively 92 ± 3% and 95 ± 3% for fecal water and 99 ± 6% and 83 ± 1% for whole feces.

Statistics
Results are presented as mean ± SEM (n=8). Statistics were calculated with a commercially available package (SPSS/PC + v2.0, SPSS Inc., Chicago, IL.) To see if the observed heme effects were dependent on the level of dietary calcium, two-way analysis of variance was performed. Then we tested differences between each heme group and its calcium-matched control group, between the two control groups, and between the two heme 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 Students 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
Analogous to our earlier work on bile acids (25) and on bilirubin (26), we explored whether heme and the heme-induced cytolytic factor were sensitive to precipitation by freshly formed amorphous CaPi. When buffered solutions containing 0 - 400 µM heme (pH=7.0) were coincubated with CaPi (10 mM), heme was completely precipitated (Figure 1). When fecal water, obtained from an earlier study with heme-fed rats (5), was preincubated with freshly prepared CaPi (10 mM) for 2 hours and subsequently centrifuged, cytotoxicity in the remaining supernatant drastically decreased, when compared with samples devoid of CaPi (Figure 2).

These in vitro results prompted us to study whether CaPi also attenuates the deleterious effects of heme in vivo. Therefore, rats were fed diets that differed only in heme and CaPi content. Food intake did not depend on the presence of heme in the diet, but was
Figure 1. Effect of CaP₁ on the solubility of heme in vitro. Heme (0-400 µM) was incubated in buffer (pH=7.0) in the absence (filled circles) or presence (open circles) of 10 mM freshly formed CaP₁. Results are given as mean of three separate experiments (the SEM’s are smaller than the size of the symbols).

Figure 2. Effect of freshly formed CaP₁ on cytotoxicity of fecal water in vitro (means ± SEM, n=8). Fecal water of control rats and of heme-fed rats was incubated in the absence (filled bars) or presence (hatched bars) of 10 mM CaP₁.
Table 1. Effect of dietary CaP₃ and heme on daily food intake, animal growth, and fecal characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Low-calcium diet</th>
<th></th>
<th>High-calcium diet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>heme</td>
<td>control</td>
<td>heme</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>17.0 ± 0.3</td>
<td>16.4 ± 0.4</td>
<td>18.1 ± 0.5</td>
<td>17.7 ± 0.4</td>
</tr>
<tr>
<td>Growth, g/day</td>
<td>3.5 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>3.7 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Fecal output (dry), g/day</td>
<td>0.61 ± 0.02ᵃ</td>
<td>0.69 ± 0.04ᵃ</td>
<td>1.55 ± 0.07ᵇ</td>
<td>1.64 ± 0.06ᵇ</td>
</tr>
<tr>
<td>Fecal cations, µmol/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- total</td>
<td>254 ± 20ᵃ</td>
<td>964 ± 31ᵇ</td>
<td>192 ± 26ᵃ</td>
<td>313 ± 18ᶜ</td>
</tr>
<tr>
<td>- sodium</td>
<td>50 ± 6ᵃ</td>
<td>487 ± 21ᵇ</td>
<td>34 ± 5ᵃ</td>
<td>125 ± 13ᶜ</td>
</tr>
<tr>
<td>- potassium</td>
<td>82 ± 8ᵃ</td>
<td>267 ± 7ᵇ</td>
<td>33 ± 6ᶜ</td>
<td>67 ± 6ᵈ</td>
</tr>
<tr>
<td>- ammonium</td>
<td>122 ± 8ᵃ</td>
<td>211 ± 18ᵇ</td>
<td>125 ± 20ᵃ</td>
<td>121 ± 6ᵃ</td>
</tr>
<tr>
<td>Wet weight (%)</td>
<td>62 ± 2ᵃ</td>
<td>86 ± 1ᵇ</td>
<td>54 ± 3ᵃ</td>
<td>67 ± 1ᶜ</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (n=8). Percentage water of feces 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 feces. Values in the same row not sharing the same superscript are significantly different (p<0.05).

slightly higher on both high-calcium diets (Table I). During the experiment, a small, nonsignificant reduction of growth was seen in the heme-fed rats. Daily fecal output of dry matter was not affected by heme 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 feces in the low-calcium heme group, we quantified the hydration of the feces by measuring the total amount of sodium, potassium, and ammonium. On the low-calcium diet, supplemental heme drastically increased the concentration of total fecal cations, and consequently, the percentage wet weight of the feces. This was largely due to the tenfold increase in fecal sodium content. Potassium and ammonium content of the feces were raised three- and twofold, respectively, by dietary heme. These heme-induced increases in fecal cations and fecal wet weight were counteracted by addition of calcium phosphate to the diet, though not completely.

We suggested before that the deleterious effects of heme might be mediated by luminal degradation or conversion of the ingested heme (5). Table II shows that the low- and the high-calcium heme group had a comparable daily intake of heme. Daily fecal output of heme was low on both control groups. When the diet was supplemented with heme, excretion of heme rose substantially on both calcium levels, and this increase was larger in the high-calcium...
Table 2. Effect of dietary CaPi and heme on heme intake and on fecal heme parameters.

<table>
<thead>
<tr>
<th></th>
<th><strong>Low-calcium diet</strong></th>
<th><strong>High-calcium diet</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>heme</td>
</tr>
<tr>
<td>Intake heme, µmol/day</td>
<td>0</td>
<td>21.4 ± 0.5</td>
</tr>
<tr>
<td>Fecal output heme, µmol/day</td>
<td>0.2 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.0 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metabolized heme, µmol/day</td>
<td>0</td>
<td>10.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heme in fecal water, µM</td>
<td>6 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM (n=8). Heme intake was calculated by multiplying daily food intake by the heme content of the diet. Heme in fecal samples was determined by a modified HemoQuant assay. The amount of metabolized heme was calculated by subtracting fecal heme output from daily intake of heme. Values in the same row not sharing the same superscript are significantly different (p<0.05)

Figure 3. UV-visible absorbance spectrum of a lipid extract of pooled fecal waters of the different dietary groups. (- - - - ); low-calcium control; (-----) low-calcium heme; (- - - - ) high-calcium control; (-----) high-calcium heme.
group. Consequently, less heme was metabolized during gastrointestinal transit in rats on a high-calcium diet.

When preparing the fecal waters, we noted a large difference in color. The fecal water of the low-calcium heme group was dark-brown, whereas the fecal waters of the other groups, including the high-calcium heme group, had a light-brown appearance. We quantitated this by recording the absorption spectra of chloroform-methanol extracts of the fecal waters (Figure 3). Interestingly, fecal water of the low-calcium heme group showed a high absorbance at 400 nm, suggesting that heme was solubilized in the aqueous phase of the feces. The much lower absorbance in fecal water of the high-calcium heme group indicates that this does not occur on the high-calcium diet. This prompted us to quantify heme in fecal water. Indeed, the concentration of heme was low in fecal water of the low-calcium control group, and even lower in the high-calcium control group (Table II). Addition of heme substantially increased the amount of heme in fecal water on the low-calcium diet, but only minor amounts were solubilized on the high-calcium diet.

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

![Figure 4](image)

**Figure 4.** Effects of dietary heme and CaPi on the cytolytic activity of 20 µL fecal water (mean ± SEM, n=8). Hatched bars, fecal water of control rats. Filled bars, fecal water of heme-fed rats. An asterisk indicates a statistically significant difference between heme-fed rats and their calcium-matched controls (P<0.001).
The effect of the diet on the proliferative activity of the colonic epithelial cells was similar to that on the cytotoxicity of the fecal water (Figure 5). There was no difference in proliferative activity between low- and high-calcium control groups. Dietary heme 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).

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

**DISCUSSION**

To our knowledge, an interaction between CaP, and heme concerning the solubility and metabolism of the latter has not been documented before. Our in vitro experiments showed...
that freshly formed CaP\textsubscript{i} binds to and precipitates the native heme molecule. In addition, CaP\textsubscript{i} diminished cytotoxicity of fecal water of heme-fed rats, indicating that CaP\textsubscript{i} also bound the heme-induced cytotoxic factor. In vivo, dietary calcium also inhibited heme-induced cytotoxicity of the fecal water, and consequently, prevented the hyperproliferation of the colonic mucosa. 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, 28).

We showed before that bile acids, fatty acids or heme itself were not responsible for the deleterious effects of dietary heme (5). Instead, we suggested that an unknown cytolytic factor was formed during the gastrointestinal passage of heme. Table II shows that both the low and high-calcium heme groups consumed the same amount of heme. Recovery of dietary heme in feces of rats consuming the low-calcium diet was about 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 approximately 10\% of the daily intake (5) our data indicate that on the low-calcium diet substantial intestinal heme metabolism must have taken place. Increasing the calcium content of the diet and the subsequent precipitation of heme in the intestinal lumen, analogous to the observed in vitro effects (Figure 1), may have resulted in a decrease of the dietary heme available for metabolism. Interestingly, consumption of the high-calcium diet also diminished the solubilization of heme compounds in the fecal water and protected against the heme-induced cytotoxicity of the fecal 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 heme. The structural elucidation of the heme-induced cytotoxic factor might reveal the underlying mechanism, and this will be the subject of future research.

To be noted the effect of dietary calcium on fecal recovery of heme may limit the diagnostic value of the HemoQuant method for the detection of intestinal bleeding. Our results indicate that the large variance in fecal recovery of dietary heme in humans (29) 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 fecal water. We have shown earlier that lytic effects of surfactants on erythrocytes and on Caco-2 cells were very similar (21) and that the cytolytic activity of fecal 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 heme-induced cytolytic activity of fecal 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 fecal stream, resulting in less epithelial damage. This is substantiated by the observation that dietary CaP\textsubscript{i} also inhibited the heme-induced increase in fecal sodium and potassium. Increasing concentrations of luminal sodium and potassium may be indicative of surfactant-induced damage to the epithelium (30, 31). Obviously, the protective effect of dietary CaP\textsubscript{i} on fecal cytolytic activity and epithelial damage leads to a normalization of the proliferative activity of the colonic epithelium (Figure 5), and thus may be relevant for the prevention of colon cancer (6, 7, 28).
In contrast with the detrimental effects of heme on the colonic epithelium, the small intestine is not affected by dietary heme, even when CaP content of the diet is low. Perhaps formation of the cytotoxic factor does not occur in the small intestine, but only in the colon, where bacterial density is high. Regardless of the mechanism, dietary heme obviously exerts its detrimental effects only in the colon. Whether this contributes to the high incidence of tumors in the colon, compared to the small intestine, is not clear.

The inhibiting effect of dietary calcium on heme-induced colonic cytotoxicity and hyperproliferation may explain why red meat does not affect cancer risk in earlier rat studies (32-34). In these studies red meat was compared with a control diet containing a relatively high concentration of calcium. Our low-calcium control diet contained 20 µmol calcium/g dry weight mimicking a daily intake of 400 mg calcium and 500 gram dry weight in humans.

Because our diets mimicked the composition of human western-style diets, our results may have implications for the human situation. No studies concerning the effects of heme on cytolytic activity of fecal 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 cytolytic surfactants (such as bile acids, fatty acids, and bilirubin, which is a heme metabolite) is similar in rats and humans (10, 15). It is therefore reasonable to assume that CaP can also bind heme or heme-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, 35-38). 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, 20), decreased cytotoxicity of fecal water. In addition, several studies (11, 13, 14), but not all (39-41), 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 (42) or calcium supplements (43) were associated with a lower risk of recurrent adenomas. Stronger evidence comes from the calcium-intervention study of Baron et al., in which supplemental calcium decreased adenoma recurrence by approximately 25% (37). In addition in the European intervention study, a modest, though not statistically significant, preventive effect of calcium was reported (44). In the study of Baron et al. however, supplemental calcium did not inhibit the epithelial proliferation effect in colonic biopsies (45), 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 (18, 19). Epithelial hyperproliferation in vivo certainly is a risk factor for carcinogenesis due to its intrinsic property to increase random
Diet-dependent effects

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Colonic lumen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red meat</td>
<td>Heme</td>
</tr>
<tr>
<td>Ca + P</td>
<td>CaP_i</td>
</tr>
</tbody>
</table>

Response of colonic epithelium

<table>
<thead>
<tr>
<th>Normal cells</th>
<th>Cell death</th>
<th>Hyperproliferation</th>
<th>Mutagenesis</th>
<th>Carcinogenesis</th>
</tr>
</thead>
</table>

Figure 6. Proposed mechanism for the interaction between dietary calcium phosphate and heme in the colonic lumen and its effect on the colonic epithelium.

mutation rate above its natural background (7, 28). Therefore, we think that the protective effect of CaP_i on the heme-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 CaP_i diet precipitates dietary heme and thus prevents the formation and solubilization of the heme-induced cytolytic factor in the fecal water. Consequently, the heme-induced increase in cytolytic activity of fecal 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 heme on the colonic mucosa, dietary calcium phosphate may contribute to the protection against colon cancer.

ACKNOWLEDGEMENTS

The authors wish to thank Bert Weijers, Maria Faassen-Peters and Annelies Landman (Small Animal Research Center, Wageningen University, Wageningen, The Netherlands) for their skillful biotechnical assistance.
REFERENCES


