Red meat and colon cancer: dietary heme, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium

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

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ABSTRACT

High intake of red meat is associated with increased colon cancer risk. It is suggested that fat from red meat is responsible because high fat intake increases the concentration of cytotoxic lipids in the colon. Experimental studies did not unequivocally support such a role for fat. Recently, we showed that dietary heme, abundant in red meat, increased colonic cytotoxicity and epithelial proliferation. In this study, we wanted to clarify whether dietary fat affects colon cancer risk by itself or by modulating the detrimental effects of heme on the colonic epithelium. Rats were fed control or heme-supplemented diets with 10, 25 and 40% energy derived from fat for 14 days. Feces were collected for biochemical analyses. Colonic cytotoxicity was determined from the degree of lysis of erythrocytes by fecal water. Colonic epithelial proliferation was measured in vivo using [3H]-thymidine incorporation. Increasing the fat content of the control diets stimulated fecal disposal of both fatty acids and bile acids. Furthermore, it increased the concentration of fatty acids, but not of bile acids, in the fecal water in control rats. Cytolytic activity of the fecal water and colonic epithelial proliferation were unaffected. Dietary heme increased fecal cation content and cytolytic activity of the fecal water at all fat levels, suggesting that the colonic mucosa was exposed to high amounts of luminal irritants. This effect was smaller on the low-fat diet. Dietary heme also increased colonic epithelial proliferation at all fat levels. The heme-induced effects were independent of fatty acids or bile acids in the fecal water. Because in Western societies 30-40% of the ingested energy is supplied by dietary fat, our results suggest that the association between red meat consumption and colon cancer risk is mainly due to its heme content, and is largely independent of the dietary fat content.

INTRODUCTION

Colon cancer is the second most common cause of cancer deaths in Western societies. It is an age-related disease, caused by a time-dependent accumulation of mutations in tumor suppressor genes and oncogenes, resulting in the subsequent transformation of normal epithelium into hyperproliferative tissue, adenoma and finally carcinoma (1). The predominant type of point mutation found in several genes of sporadic colon tumors is a G:C to A:T transition (2, 3). These mutations can cause defective protein functioning, like for instance for the APC (3) or p53 protein (4). C-to-T transitions are probably the result of endogenous processes, and may not be caused by dietary mutagens (1). In this respect, the presence of irritants in the colonic lumen is important, because these compounds can cause damage to the colonic mucosa and stimulate the continuous regeneration of the epithelium (5), which enhances endogenous mutation risk (1, 6). Bile acids and fatty acids are considered to be the main irritants in the fecal stream, and are thought to link the high-risk Western style diet to colon cancer (7). The common hypothesis purports that the high saturated fat content of this diet causes an increased excretion of bile acids and fatty acids. However, several experimental
studies failed to show that dietary saturated fat enhanced fecal bile acid excretion (8-10). Moreover, in a review of several large prospective cohort studies, colon cancer risk was associated with red meat consumption but not with total or animal fat intake (11). The authors of this review suggested that the risk-increasing effect of red meat could be due to either its iron or its fat content. To our knowledge, the interaction between red meat and fat has never been investigated in an experimental study.

Recently, we hypothesized that the association between red meat and colon cancer could be explained by the presence of heme in red meat (12). As an alternative to other hypotheses concentrating on heterocyclic amines and nitroso-compounds (see (13)), we proposed that heme and its degradation products have cytotoxic effects in the colonic lumen and thus may induce a compensatory hyperproliferation of the colonic epithelium. This hyperproliferation is commonly regarded as a risk factor for colon cancer (6). When heme was given as a dietary component to rats, we found a marked increase of fecal water cytolytic activity and of the proliferative activity of the colonic epithelium (12). Heme is an amphiphilic molecule, and its cytotoxic effects may depend on other lipid-like components of the diet. Because there is doubt whether dietary fat increases fecal fatty acids or bile acids, we propose that fat may affect the colonic epithelium by modulating the detrimental effects of heme. We therefore hypothesized that increasing the fat content of control diets (without heme) does not affect the colonic epithelial proliferation, but that it enhances heme-induced fecal water cytolytic activity and colonic epithelial cell proliferation. To study this, rats were fed control or heme-supplemented diets with either 10, 25 or 40% energy derived from fat. The fatty acid composition of the blend of fat (82% palm oil and 18% corn oil) mimicked the ratio of saturated to monounsaturated to polyunsaturated fatty acids (2:2:1) in a Western human diet (14, 15). Here we show that irrespective of the dietary fat content, heme increased cytolytic activity of fecal water and colonic epithelial proliferation, whereas fat itself had no effects. This suggests that heme, but not fat is primarily responsible for the association between red meat consumption and colon cancer.

MATERIALS AND METHODS

Animals and diets

The animal welfare committee of the Wageningen University, Wageningen, The Netherlands approved the experimental protocol. Nine-week-old male, outbred Wistar rats (Harlan Horst/Wu, The Netherlands, specific pathogen free), mean body weight 280g, were housed individually 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). For two weeks, 6 groups of 8 rats were fed purified diets, differing only in heme content and in amount of fat. The percentage of energy supplied by fat was 10%, 25% or 40% (the diets contained 42, 115 and 203 g fat/kilogram, respectively, which was exchanged for dextrose in the diet). Because the energy density of the diets increased with their fat content, the amount of all
Table 1. Composition of the diets (g/kg).

<table>
<thead>
<tr>
<th>Component</th>
<th>Low fat control</th>
<th>Low fat haem</th>
<th>Medium fat control</th>
<th>Medium fat haem</th>
<th>High fat control</th>
<th>High fat haem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>219</td>
<td>219</td>
<td>242</td>
<td>242</td>
</tr>
<tr>
<td>Dextrose</td>
<td>689</td>
<td>689</td>
<td>591</td>
<td>591</td>
<td>472</td>
<td>472</td>
</tr>
<tr>
<td>Fat</td>
<td>42</td>
<td>42</td>
<td>115</td>
<td>115</td>
<td>203</td>
<td>203</td>
</tr>
<tr>
<td>CaHPO₄.2H₂O</td>
<td>3.44</td>
<td>3.44</td>
<td>3.76</td>
<td>3.76</td>
<td>4.16</td>
<td>4.16</td>
</tr>
<tr>
<td>Cellulose</td>
<td>20</td>
<td>20</td>
<td>21.9</td>
<td>21.9</td>
<td>24.2</td>
<td>24.2</td>
</tr>
<tr>
<td>Haem</td>
<td>--</td>
<td>0.82</td>
<td>--</td>
<td>0.90</td>
<td>--</td>
<td>0.99</td>
</tr>
<tr>
<td>Ferric citrate.3H₂O</td>
<td>0.38</td>
<td>--</td>
<td>0.42</td>
<td>--</td>
<td>0.45</td>
<td>--</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35.0</td>
<td>35.0</td>
<td>38.3</td>
<td>38.3</td>
<td>42.3</td>
<td>42.3</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10.0</td>
<td>10.0</td>
<td>10.9</td>
<td>10.9</td>
<td>12.1</td>
<td>12.1</td>
</tr>
<tr>
<td>Energy (MJ/kg diet)</td>
<td>16.02</td>
<td>16.02</td>
<td>17.53</td>
<td>17.53</td>
<td>19.37</td>
<td>19.37</td>
</tr>
</tbody>
</table>

The composition of the vitamin and mineral mixtures is according to the recommendation of the American Institute of Nutrition 1993, except that calcium was omitted, tri-potassium citrate was added instead of KH₂PO₄, providing the same amount of potassium, and choline was added as choline chloride. The percentage of energy supplied by fat was 10%, 25% or 40% for the low-, medium- and high-fat diet, respectively.

Ingredients (except fat and dextrose) added to the diets was adjusted to provide equal nutrient density (g/kJ) in all diets. The composition of the diets is given in Table I. Low-, medium- and high-fat diets were supplemented with 1.26, 1.39 and 1.52 mmol heme (hemin, Sigma-Aldrich Chemie, St. Louis, MI, US) or ferric citrate (controls) (BDH, Brunswig Chemie, Amsterdam, The Netherlands) per kilogram diet, respectively. Calcium was added as CaHPO₄.2H₂O (Fluka Chemie, Buchs, Switzerland). Other minerals, vitamins, and choline as choline chloride (adjusted for energy density of the diets) were added to the diets according to the recommendations of the American Institute of Nutrition 1993 (16). Food and demineralized drinking water were supplied ad libitum. Food intake and body weights were recorded every 2-4 days. Feces were collected quantitatively during days 11-14 of the experiment and were frozen at -20°C.

**In vivo colonic epithelial cell proliferation**

After the rats had been fed the experimental diets for 14 days, DNA and protein content of the colonic scrapings and the proliferative activity of the whole colonic epithelium, quantified by the incorporation of [*methyl-³H*]thymidine (Amersham International, Amersham, UK) per µg DNA, were determined as described before (12).
**Fecal water preparation**

Feces were freeze-dried after collection. Fecal water was prepared by reconstituting the homogenized freeze-dried feces with appropriate amounts of double-distilled water to provide samples with an osmolarity of 300 mosmol/L, as described previously (12). Fecal waters were stored at –20°C until analysis.

**Cytolytic activity of fecal water**

Cytolytic activity of fecal water was quantified by potassium-release from erythrocytes, as described by Govers et al. (17), with some modifications. For this purpose, 10 and 20 µL of fecal water were mixed with saline to a volume of 80 µL. After preincubation for 5 min at 37 °C, 20 µL of a washed human erythrocyte suspension were added (final hematocrit 5%) and incubated for 15 min at 37 °C. Cytolytic activity was measured as described before (17). The relevance of this bioassay with erythrocytes for effects on intestinal epithelial cells is 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 (18).

**Fecal water analyses**

For determination of free fatty acids and bile acids in fecal water, acidified fecal water (final HCl concentration 1M) was extracted three times with 5 volumes of diethyl ether. The diethyl ether phase was dried under nitrogen and the extract was resolubilized in ethanol. Free fatty acids were determined using a colorimetric enzymatic assay (NEFA-C kit, Wako Chemicals, Neuss, Germany) and bile acids were measured with a fluorescent enzymatic assay, as described earlier (19). The amount of heme in the fecal water was measured with a modified HemoQuant assay (20) using hemin as a standard. Recovery of heme and protoporphyrin was 95 ± 6% and 83 ± 3%, respectively.

**Total feces analyses**

The amount of sodium and potassium in feces was determined using atomic emission spectrophotometry as described previously (12). Fecal ammonia was measured using the Sigma Urea Nitrogen Kit (Sigma Diagnostics, No. 640, St. Louis, MO, US), omitting the incubation step with urease (12). The percentage water of feces was calculated assuming that the total amount of sodium, potassium, ammonia and their negatively charged counterions provided an osmolarity of 300 mosmol/L in feces (21). For the determination of heme in whole feces, an acidified chloroform-methanol (22) (final HCl concentration 1M) extract was obtained from approximately 20 mg of feces. The chloroform phase of the samples was dried under nitrogen and resolubilized in 0.45 mL 250 mM KOH. Subsequently, 0.45 mL double-distilled water, 3.75 mL 2-propanol and 0.75 mL 1.15 M HCl were added. The samples were centrifuged for 10 minutes at 1500g and the supernatants were assayed for their heme content (20). Using this procedure, recovery of heme and protoporphyrin was 109 ± 7% and 95 ± 5%, respectively.
respectively. Free fatty acids and bile acids in feces were determined as described previously (5).

Statistics

Results are presented as means ± SEM (n=8). A commercially available package (Statistica 5.5 StatSoft Inc., Tulsa, US) was used for all statistics. To evaluate independent effects of increasing doses of dietary fat, one-way analysis for control groups only was performed. To test whether dietary fat interacted with effects of heme, two-way analysis of variance was performed with the fat level and the heme level of the diets as independent variables. In case of treatment effects, each heme group was compared with its fat-matched control. Distribution of data was evaluated using normal probability plots. When data were distributed normally, the Student’s t-test with Bonferroni correction was used to test for differences between means (two-sided). When data were not normally distributed, differences between the heme and their fat-matched control groups were tested using the non-parametric Mann-Whitney U test. Bonferroni correction was made for the number of equations (n=3). Differences were considered statistically significant when p<0.05 (two-sided).

RESULTS

Table II shows the effects of the different diets on daily intake of food, energy and heme and on growth of the animals. Daily food intake was lower when the amount of fat of the diet was increased (p<0.001), and this was independent of the presence of heme. However, the energy density of the diet increased with their fat content, so daily energy intake was equal among all groups. This was also the case for heme: all three heme-fed groups consumed the same amount of heme per day. Neither heme nor the fat content of the diet affected growth of the animals.

A striking observation was that the feces of rats fed the medium- and high-fat heme diets, but not the low-fat heme diet, were softened, whereas feces of rats in all three non-heme groups had a normal appearance. Because this might indicate that the composition of the diets affected colonic absorption of cations and water, we quantified this hydration of the feces by measuring the fecal concentration of cations (sodium, potassium and ammonium) (Figure 1). Fat itself had no effect on concentration of cations in the feces. In contrast, dietary heme increased fecal cation content at all three fat levels, and there was a strong interaction with the dietary fat content (p<0.001 for the interaction). Consequently, the calculated percentage wet weight of the feces was equal among all three non-heme groups, (55 ± 3%, 60 ± 2% and 59 ± 2% for the low-, medium- and high-fat diet respectively) whereas it was largely increased by the addition of heme to the diets: 73 ± 3%, 85 ± 0% and 89 ± 0% respectively.
### Table 2. Daily intake of food, energy, and heme and growth of the animals.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Food intake (g/day)</th>
<th>Energy intake (kJ/day)</th>
<th>Heme intake (µmol/day)</th>
<th>Growth (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lfat, control</td>
<td>21.7 ± 0.2</td>
<td>348 ± 4</td>
<td>0</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Lfat, heme</td>
<td>20.8 ± 0.4</td>
<td>333 ± 6</td>
<td>26.2 ± 0.5</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>Mfat, control</td>
<td>18.9 ± 0.4</td>
<td>331 ± 8</td>
<td>0</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Mfat, heme</td>
<td>19.0 ± 0.3</td>
<td>334 ± 6</td>
<td>25.9 ± 0.5</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Hfat, control</td>
<td>17.1 ± 0.3</td>
<td>331 ± 6</td>
<td>0</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Hfat, heme</td>
<td>17.1 ± 0.5</td>
<td>331 ± 9</td>
<td>26.0 ± 0.7</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (n=8). The percentage of energy supplied by fat was 10%, 25% or 40% for the low- (LFat), medium- (MFat) and high-fat diet (Hfat) respectively. Food intake and growth of the animals were recorded every 2-3 days.

![Figure 1](image-url)  

**Figure 1.** Effect of heme and increasing concentrations of dietary fat on the concentration of cations (sodium, potassium and ammonium) in the feces (mean ± SEM, n=8). The standard errors were smaller than the size of the symbols. Open circles, feces of control (non-heme) rats; filled circles, feces of heme-fed rats. Two-way analysis revealed a strong interaction between heme and the fat content of the diet on fecal cation content (p<0.001). *, Significant difference between heme-fed rats and their fat-matched controls.
Figure 2. Effect of heme and increasing concentrations of dietary fat on the colonic epithelial proliferation, determined by the incorporation of $[^{3}H]$thymidine in the mucosa (mean ± SEM, n=8). Open circles, mucosa of control (non-heme) rats; filled circles, mucosa of heme-fed rats. *, Significant difference between heme-fed rats and their fat-matched controls.

Figure 2 shows the effect of the dietary treatments on the proliferation of the colonic epithelium. Epithelial proliferation was not increased when dietary fat content of the control diets was raised from 10% energy to 40% energy. Two-way analysis of variance showed that there was an heme-induced increase in colonic epithelial proliferation, independent of dietary fat content (P<0.001 for the heme effect). The effect of heme tended to be lower on the low-fat diet. Neither heme nor fat affected the DNA or protein content or the ratio DNA/protein of the colonic scrapings compared to control values (DNA: 1029 ± 80 µg/scraping, protein: 13.0 ± 0.7 mg/scraping, ratio DNA/protein: 81 ± 7 µg/mg).

Figure 3 shows that the cytolytic activity of the fecal water of the control groups was not sensitive to variations in the fat content of the diet. In contrast, dietary fat did affect the heme-induced cytolytic activity of fecal water. When 10 µL was used in the assay, the heme-induced increase in cytolytic activity was very large on the medium- and high-fat diets, whereas this effect was much lower on the low-fat diet (p<0.001 for the interaction). When instead of 10 µL, 20 µL was used in the assay, cytolytic activity of the fecal water of the low-fat heme group was also strongly stimulated, whereas fecal water of the control groups still showed no cytolytic activity (p=0.01 for the interaction). Thus, also on a low-fat diet, heme considerably increased the cytolytic activity of the fecal water.
Figure 3. Effect of heme and increasing concentrations of dietary fat on the cytolytic activity of fecal water (means ± SEM, n=8). Open circles, 10 µL fecal water of control (non-heme) rats (cytolytic activity of 20 µL control fecal water is not shown, because it was not different from 10 µL); Filled circles (10 µL) and filled squares (20 µL), fecal water of heme-fed rats. Two-way analysis revealed a strong interaction between heme and the fat content of the diet on cytolytic activity of the fecal water (p<0.001). *, Significant difference between heme-fed rats and their fat-matched controls.

Table 3. pH, bile acids, fatty acids and heme content in fecal water.

<table>
<thead>
<tr>
<th>diets</th>
<th>pH</th>
<th>fatty acids a (mM)</th>
<th>bile acids b (mM)</th>
<th>heme c (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lfat, control</td>
<td>7.62 ± 0.06</td>
<td>1.21 ± 0.13</td>
<td>1.97 ± 0.19</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Lfat, heme</td>
<td>7.35 ± 0.13</td>
<td>2.27 ± 0.29*</td>
<td>1.68 ± 0.13</td>
<td>336 ± 31*</td>
</tr>
<tr>
<td>Mfat, control</td>
<td>7.50 ± 0.08</td>
<td>1.60 ± 0.26</td>
<td>2.16 ± 0.21</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Mfat, heme</td>
<td>6.93 ± 0.11*</td>
<td>1.46 ± 0.10</td>
<td>1.17 ± 0.11*</td>
<td>264 ± 20*</td>
</tr>
<tr>
<td>Hfat, control</td>
<td>7.35 ± 0.13</td>
<td>3.15 ± 0.61</td>
<td>2.21 ± 0.12</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Hfat, heme</td>
<td>7.19 ± 0.19</td>
<td>3.01 ± 0.49</td>
<td>0.98 ± 0.08*</td>
<td>235 ± 43*</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (n=8). a significant fat-induced increase in control (non-heme) diets. b statistically significant interaction between heme and the fat-content of the diet, p<0.05. c One-way anova revealed no significant differences in heme content of fecal water between low-, medium- and high-fat heme group. * Statistically significant difference between heme-fed rats and their fat-matched non-heme controls.
To determine the factors responsible for the diet-induced changes in fecal cytolytic activity, we analyzed the composition of the fecal water (Table III). When control groups were considered first, it appeared that the pH of the fecal water was not significantly affected by increasing the fat content of the diet. Dietary fat increased the concentration of fatty acids (p<0.001), but not of bile acids, in fecal water of controls. The heme content of control fecal waters was very low, with no differences between the dietary fat levels. When heme was added to the diets, the pH decreased at all fat levels (p=0.002), but this did not depend on the fat content of the diet. Although the concentration of fatty acids was increased by dietary heme on the low-fat diet, there was no significant overall heme-induced increase in fatty acid concentration. The bile acid concentration in fecal water was lowered by dietary heme on the medium- and high-fat diets, but not on the low-fat diet (p=0.008 for the interaction). Addition of heme to the diet increased the heme content of fecal water drastically (p<0.001), without significant differences between the fat levels.

Dietary fat did not induce a significant increase in daily fecal dry weight in rats fed the control diets (Table IV). In these groups, increasing the fat content of the diets significantly increased daily fecal output of both fatty acids (p<0.001) and bile acids (p=0.001).

**Table 4.** Effect of diets on daily fecal output of dry weight, heme, fatty acids, and bile acids.

<table>
<thead>
<tr>
<th>diets</th>
<th>dry weight$^a$ (g dry wt./day)</th>
<th>fatty acids$^{a,b}$ (µmol/day)</th>
<th>bile acids$^{a,b}$ (µmol/day)</th>
<th>heme$^c$ (µmol/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lfat, control</td>
<td>0.60 ± 0.02</td>
<td>90 ± 12</td>
<td>10.7 ± 0.6</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Lfat, heme</td>
<td>0.64 ± 0.04</td>
<td>143 ± 19</td>
<td>15.1 ± 1.3*</td>
<td>15.3 ± 1.2*</td>
</tr>
<tr>
<td>Mfat, control</td>
<td>0.66 ± 0.03</td>
<td>155 ± 13</td>
<td>13.1 ± 1.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Mfat, heme</td>
<td>0.73 ± 0.05</td>
<td>181 ± 21</td>
<td>15.3 ± 1.0</td>
<td>12.8 ± 1.0*</td>
</tr>
<tr>
<td>Hfat, control</td>
<td>0.66 ± 0.02</td>
<td>175 ± 15</td>
<td>17.0 ± 1.3</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Hfat, heme</td>
<td>0.78 ± 0.05</td>
<td>231 ± 32</td>
<td>16.5 ± 1.3</td>
<td>12.4 ± 1.2*</td>
</tr>
</tbody>
</table>

Values represent means ± SEM (n=8). $^a$ Two-way anova showed fat-independent effect of dietary heme on dry weight output (p=0.02), on fecal excretion of fatty acids (p=0.008) and bile acids (p=0.03). $^b$ Significant increase in fatty acids (p<0.001) and in bile acids (p=0.001) due to dietary fat in control (non-heme) groups. $^c$ One-way anova for the heme groups revealed no significant differences in fecal heme output between low-, medium- and high-fat heme groups. $^*$ Statistically significant difference between heme-fed rats and their fat-matched controls.
Heme output was very low in these groups, and did not depend on the fat content of the diet. Adding heme to the diets increased daily fecal dry weight excretion (p=0.02), independent of dietary fat content. It also stimulated daily fecal output of fatty acids (p=0.008) and of bile acids (p=0.03) in the feces, and for both components there was no significant interaction with fat. Fecal output of heme was slightly higher on the low-fat diet than on the medium- and high-fat diets, but this did not reach statistical significance. Metabolism of heme, calculated as the amount of ingested heme minus fecal output of heme, was 10.8 ± 1.1, 13.1 ± 0.7 and 13.6 ± 1.0 µmol/d for the low-, medium- and high-fat heme diets respectively. These differences were not significantly different from each other.

**DISCUSSION**

As mentioned in the introduction, there were two main questions to be answered in this study. First, does feeding diets with increasing fat content affect the proliferative activity of rat colonic epithelium and, secondly, does fat modulate the deleterious effects of dietary heme? To answer the first question, only control diets without heme were considered. The results of this study do not support the hypothesis that dietary fat by itself is involved in the pathogenesis of diet-induced colorectal cancer. The generally accepted hypothesis states that higher intake of fat causes an increased excretion of fatty acids and bile acids into the colonic lumen, where they can be cytotoxic to the colonic epithelium (7). In accordance with this proposed mechanism, increasing the fat content of control diets in the present study indeed enhanced the fecal excretion of these lipids. However, as shown by Lapréd et al. (23), only water-soluble surfactants, and not their concentrations in whole feces, determine the cytolytic activity of fecal water. Enhancing the fat content of control diets increased the concentration of fatty acids, but not of bile acids, in fecal water. Apparently this was insufficient to affect the colonic mucosa, because the fecal waters from all non-heme groups had very low cytolytic activity. This indicates that in the present study, dietary fat does not change the potency of the fecal water to damage the colonic epithelium. This is supported by the observation that increasing the fat content of control diets did not affect fecal cation content. High fecal cation content could be the result of luminally induced damage to the colonic mucosa (24-26). In the present study, in accordance with the lack of effect on cytolytic activity of the fecal water, the proliferation of the colonic mucosa was not affected. Thus, although fecal excretion of bile acids and fatty acids was increased by dietary fat, their impact on colonic epithelium was minimal.

In literature, there is no consistent evidence for the hypothesis that dietary fat increases colon cancer risk through an enhanced excretion of cytotoxic lipids to the colonic lumen. In animal studies, colonic epithelial proliferation was not affected (27) or was even decreased (28) by high-fat diets. Fecal bile acid excretion was stimulated by high-fat diets in some (29, 30), but not all (31) studies. Human experimental data with regard to the fat-bile acid hypothesis are limited. When studying the effects of different amounts of dietary fat on fecal bile acids, no convincing evidence was obtained for a stimulating effect of fat on fecal bile acid
excretion (8-10). Only one study has assessed the effect of increasing doses of dietary fat on colonic epithelial proliferation in humans (32). In that study, dietary fat increased proliferation of the colonic epithelium only when it was given as a single fat bolus, 6 hours after the last meal, but not when the same amount of fat was consumed during the meals.

In contrast with the lack of effect of dietary fat, heme increased the proliferative activity of the colonic epithelium. Like fat, heme increased the fecal excretion of fatty acids and also of bile acids on the low-fat diet. The reason for this is however unknown and the results indicate that the heme-induced increase of these cytotoxic lipids is not relevant for the deleterious effects of heme. The present study confirms the proposed mechanism through which heme exerts its effects on colonic epithelium (12). Consumption of heme-containing diets strongly increased the cytolytic activity of the fecal water. Together with the increase in fecal cation content, this suggests that the colonic mucosa was damaged when rats were fed the heme-supplemented diets. We have shown earlier (5) that cytotoxicity of fecal water and epithelial damage were highly correlated with the colonic epithelial proliferation, suggesting that loss of cells is compensated by an increased proliferative activity of the colonic epithelium. Indeed, consumption of heme-containing diets by the rats increased the colonic epithelial proliferation.

Though dietary heme strongly increased fecal water cytolytic activity, it did not increase the concentration of fatty acids in fecal water. In addition, bile acid concentration in the fecal water of heme-fed rats was even lower compared to controls, probably due to dilution by the increased amount of water in the feces. We also measured the pH of the fecal waters, because red blood cells in the bioassay are sensitive to variations in the pH of the incubation medium. However, simple acid-induced lysis can be excluded, because the pH of fecal waters from the heme-supplemented groups was within the pH range of controls. These results indicate that a hitherto unknown, highly cytolytic factor was formed in the intestine of heme-fed rats, which can be responsible for the mitogenic effect of dietary heme. To be noted, we showed before that the cytolytic activity of the fecal water and the colonic epithelial proliferation was increased only when heme was present in the diet, but not when heme was replaced by equimolar amounts of its separate constituents inorganic iron or protoporphyrin (12). This indicates that the presence of iron is a prerequisite for the formation of the cytolytic factor, but only when it is in the form of heme. This is in contrast with the study of Lund et al. (33), who reported a small increase in the number of mitotic cells in rat colonic crypts when the iron content of the diet was increased 3.5 times using ferric sulfate.

The second main question of this study was whether the effects of heme were dependent on the fat content of the diet. The heme-induced cytolytic activity was lower on a low-fat diet, suggesting that in these rats, exposure of the colonic mucosa to luminal irritants was less. In addition, the heme-induced increase in fecal cation content, reflecting epithelial damage, was strongly fat-dependent. The ultimate physiological effect through which heme is supposed to increase colonic cancer risk, is by increasing damage to epithelial cells. As a consequence, dietary heme at all fat levels significantly increased the proliferative activity of the colonic epithelium, although the effect of heme was slightly less on the low-fat diet. To be
noted, the concentration of dietary heme in the present study is relatively high compared with concentrations that can be expected in the human situation (34). If lower concentrations of dietary heme exert similar effects as described in the present study, it cannot be excluded beforehand that these effects are not affected by variations in the dietary fat content. A recent clinical trial considered the effect of a reduction in dietary fat, in combination with an increase in fiber, on the recurrence of colorectal adenomas. Against an almost stable background of red meat intake, and thus heme intake, no protective effect was found when the contribution of fat to total energy intake was decreased from 36% to 24% (35).

Summarizing, this study shows that increasing the dietary fat content from 10% energy to 40% energy in the absence of heme does not affect the cytolytic activity of colonic contents and epithelial proliferation, whereas both parameters are increased by dietary heme. The effect of heme on proliferation was slightly lower on the low-fat diet, corresponding with a smaller heme-induced increase in cytolytic activity of fecal water. Considering that in Western societies 30-40% of the ingested energy is supplied by dietary fat (14, 15), our results suggest that the epidemiological association between red meat consumption and colon cancer risk is mainly due to heme, and is unlikely to be dependent on the fat content of the diet.

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