Red meat and colon cancer
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CHAPTER 6

Purification and characterization of a novel, dietary heme-induced factor that has cytotoxic and hyperproliferative effects on rat colonic epithelium

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

Dietary heme increases the cytolytic activity of fecal water and proliferation of the colonic epithelium. In this study we isolated and partially characterized the heme-induced cytolytic factor of fecal water. The heme-induced cytolytic activity of fecal water could be completely extracted with chloroform/methanol. When these lipid extracts were subjected to reversed-phase chromatography, cytolytic activity co-eluted with a large bulk of heme-containing material that could not be separated into discrete compounds. Using size exclusion chromatography, cytolytic activity was confined to a heme-containing, high-molecular weight fraction that was free of bile acids and fatty acids. The heme-moiety of the high-molecular weight factor had modified vinyl side chains and it still contributed to the cytolytic activity of the compound. Activity was inhibited by the lipopolysaccharide (LPS) binding antibiotic polymyxin-B-sulfate, suggesting the presence of LPS. However, no LPS-specific esterified (hydroxy) fatty acids could be detected in the high-molecular weight fraction. The cytolytic activity of the heme-induced factor was not affected by heat treatment and pronase. Preliminary results of element analysis showed that the sulfur content of the high-molecular weight fraction of heme fecal water was at least tenfold higher than that of controls. In addition, in contrast to controls, the factor was stained in a protein assay. It therefore can be speculated that the cytolytic heme factor consists of heme molecules covalently modified at their vinyl groups by hydrophobic, pronase-resistant (poly)peptides.

INTRODUCTION

Colon cancer is a disease of the colonic mucosa, where the normal tissue homeostasis is disrupted by an age-related clonal accumulation of multiple mutations in several cell cycle control genes (1). In literature, there is ample evidence that this process is strongly influenced by environmental factors, in particular diet (2). How dietary components deregulate the normal tissue homeostasis of the colonic epithelium is not precisely known. Based on mutational analysis of colorectal carcinomas, Kinzler and Vogelstein (3) argued that constant luminal irritation of the colonic mucosa due to the consumption of high-risk diets is probably more important than the involvement of dietary mutagens. Because of this luminal irritation, increased tissue regeneration is provoked, stimulating proliferative activity (4) or inhibiting apoptotic rate of colonic epithelial cells (5). Both events are thought to increase colon cancer risk.

Epidemiologically, the incidence of colon cancer has been associated with a high consumption of red meat, but not of white meat (6, 7). We recently hypothesized that heme, the iron carrier from red meat, might be involved in this association (8). When rats were fed diets, supplemented with heme at doses that mimic the heme content of human diets in Western societies, the cytolytic activity of the fecal water was drastically increased, indicating that the colonic mucosa was exposed to large amounts of luminal irritants. As a result, the
proliferative activity of the colonic epithelium was increased by approximately 70%. Hyperproliferation is commonly regarded as a risk factor in carcinogenesis. Bile acids and fatty acids, well known luminal irritants that are thought to link high-risk diets with colon cancer, appeared not to be responsible for this heme-induced cytolytic activity (8). Heme itself was solubilized in the fecal water, but its concentration was too low to cause the high cytolytic activity. Hence, an additional, hitherto unknown highly cytolytic factor is formed in the intestine of heme-fed rats. The formation of this cytotoxic factor appeared to be highly specific for dietary heme, because other related tetrapyrroles, protoporphyrin IX and bilirubin, or inorganic iron failed to increase the cytotoxicity of fecal water and epithelial proliferation. Because red meat is by far the largest source of dietary heme (9), identification of the unknown factor in fecal samples might provide a specific biological risk marker for the consumption of red meat and reveal a novel molecular link between red meat consumption and colon cancer. In the present study, we isolated and partially characterized the unknown colonic factor, which causes the cytotoxic effects of dietary heme.

MATERIALS AND METHODS

Samples
Fed control diets or diets containing heme (with 25% of energy derived from fat), were obtained from the study in which the influence of increasing dietary fat content on the effects of heme on colonic epithelium was investigated (chapter 3) (10). In addition, feces from rats fed a diet containing protoporphyrin IX was obtained from the study of the effects of different tetrapyrroles on rat colonic epithelium (chapter 2) (8). Except for the tetrapyrrole, composition of this diet was similar to the other diets mentioned above. Preparation of fecal waters was done exactly as described before (8) and fecal waters were pooled for each group by mixing equal amounts of the individual fecal waters.

Extraction of lipids from the pooled fecal waters
Total lipid was extracted from fecal water using a chloroform/methanol extraction (11). In short, 100 µL of fecal water was mixed with 620 µL diluted HCl (final HCl concentration 1 M) and 2700 µL chloroform/methanol (1:2). After vigorous mixing, 900 µL chloroform and 900 µL double distilled water were added and the mixture was vortexed again. The lipid-containing chloroform phase was obtained after centrifugation (5 min 3000g) and evaporated under a stream of nitrogen.

Reversed-phase high performance liquid chromatography
For reversed-phase HPLC, we used a modified procedure according to Lim and Peters (12). A Waters 600E Pumping System (Waters Corporation, Milford, MA, US) was used, coupled to a UV detector (UV1000 Detector, Spectra Physics, San Jose, CA, USA) set at 400 nm to detect the Soretband of heme. Samples (dried lipid extracts of fecal waters, solubilized
in a mixture of 45:45:10 (v/v) methanol:water:acetonitrile) were injected via Waters 717 Autosampler (Waters Corporation) fitted with a 500 µL loop. The separation was performed on a 250 x 4.6 mm i.d. PLRP-S column (300Å, 8µm, Polymer Laboratories Ltd., Shropshire, UK). The solvents for the gradient elution were 10% acetonitrile in 0.1 M ammonium acetate, pH=5.2 (solvent A) and 10% acetonitrile in methanol (solvent B). The linear gradient started at 100% solvent A and changed to 100% solvent B in 25 minutes, followed by isocratic elution of solvent B for 15 minutes, using a flow rate of 1 mL/min. Data were processed with the Spectra Physics 4400 Integrator. Fractions were collected as indicated in Figure 2, evaporated in a rotating vacuum evaporator and lipids were extracted using the chloroform/methanol extraction as described above. Subsequently, these samples were tested for cytolytic activity. Hemin (Sigma-Aldrich Chemie, St. Louis, MI, US) was used as a standard for the chromatographic procedure.

Size exclusion chromatography

A Waters 501 Pumping System (Waters Corporation, Milford, MA, US) was used, in combination with a UV detector (Applied Biosystems, 757 Absorbance Detector, Ramsey, NJ, US) set at 400 nm. Samples, dried lipid extracts of 100 µL fecal water were solubilized in 250 µL of the elution solvent. Then, 200 µL were injected via a Gilson 231 Autosampler (Gilson Medical Electronics Inc. Middleton, US) fitted with a 500 µL loop. The sample was isocratically separated on two serial Jordi GPC/divinylbenzene columns (300 x 7.8 mm, 5µm, 500Å, Jordi Associates, Bellingham, MA, US) using chloroform/methanol/triethylamine (70:30:0.5, v/v) as the solvent at a flow rate of 1.0 mL/min. Data were processed on the Spectra Physics 4270 Integrator. Three fractions were collected as indicated in the figure, evaporated under nitrogen and weighed. Bile acids in the fractions were determined with a fluorescent enzymatic assay, as described before (13). Non-esterified fatty acids were measured using a colorimetric enzymatic assay (NEFA-C kit, Wako Chemicals, Nuess, Germany). Hemin (Sigma-Aldrich Chemie, St. Louis, MI, US) was used as a standard for the chromatographic procedure.

Cytolytic activity assay

Dried lipid extracts or dried samples obtained after reversed-phase chromatography or size exclusion chromatography were resolubilized in a 200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (pH=7.2, osmolarity=300 mosmol/L), providing the same concentration as in the original fecal water. These samples or original fecal water were mixed with MOPS-buffer to obtain a volume of 80 µL and were incubated 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, cytolytic activity was quantified by potassium-release from erythrocytes and where indicated, it was calculated as area under the curve, as described previously (14).
Pyridine hemochrome spectrometry

Dried samples that were obtained from the size-exclusion chromatography procedure as mentioned above solubilized well in alkaline solutions. Therefore, samples were directly dissolved in 0.5 mL 100 mM NaOH and pyridine hemochrome spectra were determined according to Berry (15). For this, samples (0.5 mL) were mixed with 0.5 mL of a solution of 100 mM NaOH and 40% pyridine (by volume) and 3 µL of 0.1 M K$_3$Fe(CN)$_6$ (dissolved in 100 mM NaOH). After mixing thoroughly, the oxidized spectrum was recorded from 400-700 nm (Perkin Elmer, Lambda 2, Norwal, MO). Then, a few crystals of solid sodium dithionite were added and the mixture was shaken gently. The reduced spectrum was recorded and the reduced minus the oxidized spectrum was determined by subtraction. The experiment was performed in duplicate and identical spectra were obtained.

Miscellaneous treatments

To further characterize the high-molecular weight factor, dried samples from fraction 1 of the size exclusion chromatography (see results) were solubilized in 200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (pH=7.2, osmolarity=300 mosmol/L) and subjected to the procedures described below. After this, the remaining cytolytic activity of the samples was measured as described above.

1. Treatment with bilirubin oxidase (Sigma-Aldrich Chemie, St. Louis, MI, US) (0.5 U/mL) for 60 minutes at 37°C. To show that heme compounds were sensitive to treatment with bilirubine oxidase, hemin standard was treated with the enzyme and absorbance was measured at 400 nm. The activity of bilirubin oxidase was checked by incubating the enzyme with bilirubin. In all cases, heat-inactivated bilirubin oxidase served as control.

2. Treatment with heat for 10 minutes at 95 °C.

3. Treatment with pronase (Roche, Basel, Switzerland) (20 U/mL) for 18 hrs at room temperature. Lipids were then extracted with an acidified chloroform/methanol extraction and assayed for cytolytic activity. The activity of pronase was checked by incubating with albumin and measuring the absorbance at 280 nm of the supernatant of an trichloroacetic acid treated sample. Heat-inactivated pronase served as control.

4. Treatment with polymyxin B-sulfate (Sigma-Aldrich Chemie, St. Louis, MI, US) (0 – 0.1 mg/mL) for 10 minutes at 37°C.

Esterified fatty acid analysis of the high-molecular weight fractions

500 µL of fecal water of heme-fed and control rats was purified using the acidified chloroform/methanol extraction and size-exclusion chromatography as described above. The high-molecular weight fractions (fraction 1, approximately 1.5 mg dry weight for the heme fecal water and 0.5 mg for the control fecal water) were subsequently treated in 2 mL of 3.6 M hydrochloric acid in methanol at 100°C for 18 hrs. Samples were extracted twice with 2 mL hexane and the hexane phase was evaporated under a stream of nitrogen until the samples were almost dry (16). For derivatization of hydroxy fatty acids, 50 µL of trifluoroacetic acid anhydride and 150 µL acetonitrile were added and the samples were incubated for 2 h at
100°C (17). A volume of 0.7 µL (direct on column injection) was injected on a gas chromatograph (Carlo Erba, HRGC 5160 Mega series), equipped with a CP-Sil 5B column (20m, 0.32 mm i.d.). Hydrogen was used as the carrier gas, at an inlet pressure of 60 kPa. The temperature of the column was programmed from 80°C to 270°C at 10°C/min. A flame ionisation detector (FID) was used to analyse the derivated fatty acids. 2 mg of LPS of Salmonella minnesota was used as a control, and treated exactly as the high-molecular weight heme factor.

RESULTS AND DISCUSSION

In our previous study (8), we showed that when rats were fed heme-containing diets the cytolytic activity of the water phase of their feces was strongly increased. Results were presented showing that bile acids, fatty acids and heme itself were not responsible for this effect. We also checked the food that was given to the rats for the presence of a cytolytic factor, but an acidified chloroform/methanol extract of the food had no cytolytic activity. Thus, a hitherto unknown, highly cytolytic factor was formed in the intestine of heme-fed rats. Given the lipid-soluble character of known cytolytic agents such as bile acids and fatty acids, we expected that this unknown, heme-induced factor could also be lipid soluble.

![Figure 1](image.png)

**Figure 1.** Comparison of the dose-dependent cytolytic activity of a lipid extract of fecal water with that of original fecal water of controls and heme-fed rats (mean ± SEM of three separate experiments). Circles, samples from control rats; squares, samples from heme-fed rats; open symbols, original fecal water; filled symbols, lipid extracts of fecal water.
To study this, an acidified lipid extract of fecal water of control and heme-fed rats was obtained, and cytolytic activity of this extract was compared with that of the original fecal water (Figure 1). For the control rats, neither fecal water nor its lipid extract showed substantial cytolytic activity in our bioassay. Cytolytic activity of fecal water of heme-fed rats was very high. As predicted, this activity could be extracted completely in the lipid phase. Moreover, the cytolytic activity of the lipid phase was even higher than that of the fecal water, which suggests the presence of inhibiting substances (such as lipid-binding proteins) in the original fecal water. These observations substantiate our assumption that lipid-like compounds mediated the deleterious effects of heme. We previously showed that the concentration of heme compounds in a lipid extract of heme fecal water was largely increased compared to controls (8). Because the heme-induced cytolytic activity of fecal water could not be explained by heme itself (8), we tested for the presence of potentially cytolytic metabolites of heme by subjecting lipid extracts of fecal waters to reversed-phase chromatography.

Figure 2. Gradient elution profiles of lipid extracts of fecal water from heme-fed rats (a.), control rats (b.) and of a hemin standard (c.) on a reversed-phase column, measured by the absorbance at 400 nm. Fractions were collected as indicated in the figure and tested for cytolytic activity.
Typical elution patterns of fecal waters and of heme standard are shown in Figure 2. A hemin stock solution, which was first extracted using the modified chloroform/methanol extraction mentioned above, eluted from the column at 20 minutes (Figure 2c) and did not differ from a hemin stock solution that was directly applied to the column (not shown). The lipid extract of control fecal water was almost completely devoid of UV-absorbing material (Figure 2b). When the lipid extract of heme fecal water was applied to the column, a UV-absorbing compound eluted from the column with the same retention as the hemin standard (Figure 2a). The heme fecal water was further characterized by the presence of a large bulk of UV-absorbing material that eluted between 12 and 28 minutes, thus including more hydrophilic as well as more hydrophobic components as compared to hemin. We collected fractions as indicated in figure 2 and tested them for cytolytic activity. All fractions of the control group were devoid of cytolytic activity, as were the fractions 1 and 4 of the heme fecal water. Cytolytic activity co-eluted with the UV-absorbing material in fractions 2 and 3, but it was not confined to either the more hydrophilic (fraction 2) or the more hydrophobic components (including the compound eluting at 20 minutes, fraction 3) (data not shown). The UV-absorbing material could not be separated into discrete compounds by changing the chromatographic conditions, or by using other solvents (data not shown).

The behavior of the UV-absorbing bulk from the heme fecal water on the reversed-phase column suggests that the heme was changed in either its chemical structure or its molecular weight. To explore the latter possibility, the lipid extracts of the fecal water were subjected to size exclusion chromatography (SEC), which separates high-molecular weight from low-molecular weight compounds. Because lipids can form large micelles in aqueous solutions, the separation was performed in a hydrophobic solvent (methanol/chloroform) to keep the lipids in solution as monomers. Lipid extracts of fecal waters of rats fed control diets or diets containing heme or protoporphyrin IX were compared with standard hemin. Typical size exclusion chromatographic elution patterns of lipid extracts of fecal waters and of hemin are shown in Figure 3. To determine whether cytolytic compounds were trapped on the column, all material eluting from the column (i.e. fractions 1-3) was collected and its cytolytic activity was compared to that of the original lipid extract. Recovery of the cytolytic activity was 100%, indicating that there was no cytolytic material lost in the purification procedure. A hemin stock solution eluted from the column after 22 minutes (Figure 3c). The fecal water of controls contained little UV-absorbing material, eluting at about 15 minutes (Figure 3b). The UV-absorbing material of fecal water of heme-fed rats contained two major components, a high-molecular weight compound eluting between 10 and approximately 16 minutes and a low-molecular weight compound eluting at a time similar to standard hemin (Figure 3a). In addition, a minor compound eluting at about 17 minutes was found. The lipid extract of the fecal water of rats fed the protoporphyrin diet lacked the huge bulk of UV-absorbing material in the high-molecular weight fraction and also the UV-absorbing compounds in the low-molecular weight fraction (not shown). Subsequently, fractions were collected as indicated in Figure 3 for all samples and tested for cytolytic activity (Table 1). Cytolytic activity of the
Figure 3. Size-exclusion chromatography of lipid extracts of fecal waters from rats fed a heme-supplemented diet (a.) or control diet (b.) and of a hemin standard (c.), measured by the absorbance at 400 nm. Fractions were collected as indicated in the Figure and assayed for cytolytic activity, bile acids and fatty acids.

The high-molecular weight fraction (fraction 1) of the heme group was very high, whereas fractions 1 of the control and protoporphyrin group showed no activity. Fraction 2 of the heme group and of the control and protoporphyrin group also contained cytolytic activity, although at a lower level. Interestingly, bile acids and fatty acids were present only in fractions 2 of all fecal waters and can explain the cytolytic activity of these fractions. Irrespective of the source of fecal water, no cytolytic activity could be detected in the low-molecular weight fractions (fraction 3). Thus, using this size exclusion chromatographic procedure, we isolated a high-molecular weight factor that is responsible for the high cytolytic activity of fecal water of heme-fed rats. Approximately 0.3 mg dry weight of the cytolytic compound could be extracted from 100 µL fecal water, whereas the same fraction of the control contained about 0.1 mg dry weight. According to the specifications of the column, supplied by the manufacturer, the heme-specific cytolytic compound has an estimated molecular weight of about 5-10 kDa.
Table 1. Cytolytic activity and concentration of bile acids and fatty acids in fractions collected during size exclusion chromatography of lipid extracts of fecal water of rats fed control, protoporphyrin or heme supplemented diets (8).

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Cytolytic activity (%)</th>
<th>Bile acids (mM)</th>
<th>Fatty acids (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>porphyrin</td>
<td>heme</td>
</tr>
<tr>
<td>1</td>
<td>7 ± 1</td>
<td>9 ± 5</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>21 ± 5</td>
<td>35 ± 4</td>
<td>57 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>4 ± 2</td>
<td>1 ± 1</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>control</td>
<td>0.02 ± 0.00</td>
<td>0.08 ± 0.04</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>porphyrin</td>
<td>2.04 ± 0.10</td>
<td>2.42 ± 0.06</td>
<td>1.14 ± 0.17</td>
</tr>
<tr>
<td>heme</td>
<td>0.04 ± 0.00</td>
<td>0.05 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>bile acids (mM)</td>
<td>0.02 ± 0.00</td>
<td>1.03 ± 0.03</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>fatty acids (mM)</td>
<td>0.11 ± 0.01</td>
<td>1.11 ± 0.06</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>control</td>
<td>0.08 ± 0.03</td>
<td>1.04 ± 0.03</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM of three separate experiments. For the determination of the cytolytic activity of the fractions, increasing amounts of the collected material (0-1-2-5-10 µL) were tested in our cytolytic activity assay and the cytolytic activity was quantified as the area under the hemolytic curve, as described previously (14). Bile acids and fatty acids were measured after solubilizing dried fractions in the same volume as the original fecal water.

To test whether heme compounds were still present in the high- and low-molecular weight fractions of the fecal waters, we recorded the reduced minus oxidized pyridine hemochrome spectra (15) of these fractions, and compared these with standard hemin. This technique may give information not only about the presence of heme compounds, but may also reveal whether these hemes are structurally modified. The purification procedure itself appeared not to affect the structure of heme compounds, because hemin that was subjected to the purification procedure showed the same spectrum as untreated hemin (data not shown). Fractions 1 and 3 of control fecal water were devoid of heme compounds, because no heme-specific signal was recorded when measuring these samples. The spectrum from the low-molecular weight fraction (Figure 3, fraction 3) of the heme fecal water shows that in this fraction, heme is still present and that this heme was unchanged compared with standard heme (Soret: 419 nm (not shown), β-band: 524 nm, α-band: 556 nm). Thus, the low-molecular heme-compound in fecal water from heme-fed rats is most likely intact heme that has passed
Figure 4. Reduced minus oxidized pyridine hemochrome spectra of the low- (---) and high-molecular (-----) fractions of heme fecal water, obtained from the size exclusion chromatography (fraction 3 and 1, respectively, cf Figure 3) and compared with a stock solution of hemin standard that was subjected to the same procedure ( )

unchanged through the gastrointestinal tract. Apparently, its concentration is too low for cytolytic activity. In contrast, heme from the high-molecular weight fraction (Figure 3, fraction 1) exhibited a blue-shift of 5 nm for the $\alpha$-band and of 3 nm for the Soret (not shown) and $\beta$-band. This spectrum is very similar to that of 2(4)-vinyl-4(2)-hydroxyethyl-deuteroheme (18), in which one of the vinyl side chains of heme is hydrated. The spectrum also resembles that of heme-c (18), the heme-moiety of cytochrome c, in which both vinyl groups are covalently attached to cysteine-residues in the apoprotein.

Subsequently, we determined whether this modified heme still contributed to the cytolytic activity of the high-molecular weight fraction. Therefore, the high-molecular weight fraction was treated with bilirubin oxidase. To ensure that heme compounds are physiological substrates for bilirubin oxidase, standard hemin, which is cytolytic at millimolar concentration, was treated with the enzyme. Bilirubin oxidase decreased both the Soret band of standard hemin and inhibited its cytolytic activity, whereas heat-inactivated enzyme was ineffective (data not shown). Thus, the cytolytic activity of hemin can be inhibited by modifying its structure. When the high-molecular cytolytic factor was treated with bilirubin oxidase, cytolytic activity was substantially inhibited (Table 2), corresponding with a decrease of the Soret band (not shown). The heat-inactivated enzyme was not effective in changing
Table 2. Percentage inhibition of the cytolytic activity of the high-molecular weight fraction (obtained by the size exclusion chromatographic procedure) of heme-fed rats by treatment with bilirubin oxidase, heat or pronase.

<table>
<thead>
<tr>
<th>treatment</th>
<th>% inhibition cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>bilirubin oxidase</td>
<td>49 ± 5%</td>
</tr>
<tr>
<td>heat-inactivated</td>
<td>0%</td>
</tr>
<tr>
<td>heat</td>
<td>15 ± 1%</td>
</tr>
<tr>
<td>pronase</td>
<td>6 ± 12%</td>
</tr>
<tr>
<td>heat-inactivated</td>
<td>10 ± 9%</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM (n=3). For testing the cytolytic activity, an amount of the high-molecular weight fraction which was equivalent to 2 µL of original fecal water was used. The high molecular weight fraction of control fecal water was used as control and tested under each condition, but cytolytic activity was always 0%.

either the Soret band or the cytolytic activity. This indicates that the heme moiety of the high-molecular weight factor still contributes to its cytolytic activity.

The increased molecular size of the cytolytic factor might be explained by the formation of complexes of aggregated heme-molecules in aqueous solutions due to hydrophobic interactions. This seems however unlikely, because such interactions probably do not occur in the hydrophobic environment of the SEC-procedure. In addition, aggregated heme shows a pyridine hemochrome spectrum with absorption maxima shifting towards higher wavelengths (red shift) (19), in contrast with the blue shift in the spectrum of heme from the cytolytic factor (Figure 4). It is therefore tempting to speculate that in the intestine, heme is bound to some kind of high-molecular compound through the modification of its vinyl side chains. To further characterize the heme-induced factor, we performed additional experiments in which the cytolytic activity was used as endpoint measurement to evaluate the effects of treatments. As shown above, the heme-induced cytolytic activity could be completely extracted in the lipid phase of the fecal water. This suggests that the unknown factor has a lipid-like structure, and the presence of a proteinaceous compound is therefore less likely, but cannot be excluded. We first explored the latter possibility. When the high-molecular factor was incubated for 10 minutes at 95°C, cytolytic activity was only marginally inhibited (Table 2). In addition, treatment with pronase for 18 hours did not significantly affect cytolytic activity. Though these experiments are not totally conclusive, we felt that proteinaceous compounds were not likely to be involved in the cytolytic activity of fecal water of heme-fed rats.
An interesting observation was that the heme-induced factor, which could be extracted from the fecal water with a chloroform/methanol solution, solubilized well in the aqueous environment of the MOPS-buffer used in the cytotoxicity assay, despite the absence of bile acids and fatty acids after size exclusion chromatography. This points at an amphipathic character of the heme-induced factor. A high-molecular compound with such properties, normally present in the colonic lumen is lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria. Binding of heme to LPS has been described before (20), but the type of binding (hydrophobic vs covalent) has not been determined. However, cytolytic activity of this heme-LPS complex was not studied. We argued that if LPS would be present in the high-molecular weight fraction of fecal water of heme-fed rats, its cytolytic activity should be inhibited by polymyxin-B-sulfate (PMB), an antibiotic with known LPS-neutralizing properties (21). Figure 5 shows that the cytolytic activity of the heme-induced factor was completely inhibited at PMB-concentrations of 0.1 mg/mL, suggesting that LPS is indeed present in the high-molecular weight fraction. However, although LPS binds to PMB with high affinity, this binding is not specific for LPS. When standard hemin (500 µM) was treated with PMB (0.1 mg/mL), its cytolytic activity decreased with 33% (not shown). It can therefore be argued that PMB inhibited cytolytic activity of the heme-induced factor by binding to the heme-moiety of the factor.

Figure 5. Inhibition of the cytolytic activity of the high-molecular weight fraction of control fecal water and of heme fecal water (amount used was equivalent to 2 µL of original fecal water) by incubation with increasing concentrations of polymyxin-B-sulfate (mean ± SEM, n=3).
To test whether LPS was indeed present in the high-molecular weight fraction, this fraction was subjected to a hydrolysis procedure that liberates (hydroxy) fatty acids from LPS. The efficacy of the procedure was confirmed by the liberation of fatty acids from LPS of Salmonella minnesota (Figure 6). However, treatment of the cytolytic factor did not release substantial amounts of fatty acids, and there were no indications for the presence of LPS-specific hydroxy fatty acids (Figure 6). In addition, the cytolytic activity of the high-molecular weight factor was not affected by this hydrolysis procedure. Apart from the possibility that the unknown factor is highly resistant to hydrolysis with methanolic HCl, this suggests that LPS is not present in the high-molecular weight fraction of the fecal water of heme-fed rats.

**Figure 6.** Gas chromatographic analysis for (hydroxy-)fatty acids in the high-molecular weight fraction of fecal water of heme-fed (a.) and control rats (b.) after acid methanolic hydrolysis and derivatization with trifluoroacetic acid anhydride. LPS of Salmonella minnesota (c.) was used as control. C-12, lauric acid; C-14, myristic acid; C-14-OH, hydroxymyristic acid; C-16, palmitic acid.
To gain more insight in the elemental composition of the unknown factor, high-molecular weight fractions of control and of heme fecal water were subjected to inductively coupled plasma atomic emission spectrometry (ICP-AES). From these analyses, only preliminary results were available. Surprisingly, apart from an expected difference in iron content (60 vs 10 µM for heme and control fecal water, respectively), the amount of sulfur in the high-molecular weight fraction of fecal water of heme-fed rats was at least 10-fold higher than in controls (1.3 mM vs 0.1 mM for heme and control fecal water respectively). The high-molecular weight fraction did not contain phosphorus, manganese or copper. Additionally, compared with control, the high-molecular weight fraction of heme fecal water could be stained in a protein assay (Pierce), suggesting the presence of proteinaceous compounds. This seems contradictory to our earlier experiments, in which we showed that the heme-induced factor was refractory to heat treatment and pronase.

In conclusion, the results of the present study suggest that the cytolytic activity of the fecal water of heme-fed rats is caused by an amphipathic, high-molecular weight compound. This factor contains heme-compounds with modified side chains. Preliminary results suggest that the factor also has a proteinaceous part, but it is highly resistant to treatment with heat and pronase. It is tempting to speculate that heme is bound to a high-molecular weight sulfur-containing compound via its modified vinyl side-chains. However, the exact nature of the heme-induced cytolytic factor and its mechanism of action still needs further investigation.

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