Dietary heme induces acute oxidative stress, but delayed cytotoxicity and compensatory hyperproliferation in mouse colon

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Red meat consumption is associated with an increased colon cancer risk. Heme, present in red meat, injures the colon surface epithelium by generating cytotoxic and oxidative stress. Recently, we found that this surface injury is compensated by hyperproliferation and hyperplasia of crypt cells, which was induced by a changed surface to crypt signaling. It is unknown whether this altered signaling is caused by cytotoxic stress and/or oxidative stress, as these processes were never studied separately. The aim of this study was to determine the possible differential effects of dietary heme on these luminal stressors and their impact on the colonic mucosa after 2, 4, 7 and 14 days of heme feeding. Mice received a purified, humanized, control diet or the diet supplemented with 0.2 µmol heme/g. Oxidative and cytotoxic stress were measured in fecal water. Proliferation was determined by Ki67-immunohistochemistry and mucosal responses by whole-genome transcriptomics. After heme ingestion, there was an acute increase in reactive oxygen species (ROS) leading to increased levels of lipid peroxidation products. Mucosal gene expression showed an acute antioxidant response, but no change in cell turnover. After day 4, cytotoxicity of the colonic contents was increased and this coincided with differential signaling and hyperproliferation, indicating that cytotoxicity was the causal factor. Simultaneously, several oncogenes were activated, whereas the tumor suppressor p53 was inhibited. In conclusion, luminal cytotoxicity, but not ROS, caused differential surface to crypt signaling resulting in mucosal hyperproliferation and the differential expression of oncogenes and tumor suppressor genes.

Introduction

Colon cancer is an important health problem in Western countries (1). Diets high in red meat are associated with an increased risk of colon cancer (2–4), whereas diets high in white meat do not increase this risk (5,6). Kinzler et al. (7) argued that dietary factors increasing the risk of colon cancer are probably not mutagens, but rather luminal irritants that damage colonic epithelial cells. The iron-porphyrin pigment heme, which is such a luminal irritant, is present at much higher levels in red meat than in white meat. Several epidemiological studies show that the intake of dietary heme is associated with an increased cancer risk (8,9).

When rodents receive a diet supplemented with heme, increased reactive oxygen species (ROS) are found in the fecal water (10,11), leading to oxidative stress responses in the mucosa. In addition, the colonic contents become more cytotoxic on the heme diet (11,12). Consequently, the colonic surface epithelium is damaged and compensatory hyperproliferation is initiated in the crypts. We showed recently that dietary heme initiated this hyperproliferation and hyperplasia by downregulating feedback inhibitors of proliferation, such as Wnt inhibitory factor 1, interleukin-15 (IL-15), Indian Hedgehog (Ihh) and bone morphogenetic protein 2 in the surface epithelium (12).

The increases in ROS, cytotoxicity and proliferation were observed after 14 days of heme feeding. So far, the time dependency of the heme-induced ROS stress and cytotoxic stress on the initiation of hyperproliferation was not studied. It is currently unknown whether the described changes in surface to crypt signaling (12) were caused by cytotoxic stress and/or oxidative stress. Therefore, we now studied the time course of the possible differential luminal and mucosal effects of dietary heme. We used a heme dose of 0.2 µmol/g diet, which mimics a human red meat consumption of 160 g/day. Our results show that acute effects, which were already established within 2 days of heme feeding, were the formation of ROS and lipid peroxidation products and the activation of many peroxisome proliferator-activated receptor (PPAR) target genes. The more delayed effects, occurring after day 4 of heme feeding, were a significant increase in cytotoxicity of the colon contents and the concomitant induction of compensatory hyperproliferation and hyperplasia in the mucosa.

Materials and methods

Animals and diets

Experiments were approved by the ethical committee on animal testing of Wageningen University and were in accordance with national law. Eight week old male C57Bl/6 mice (Harlan, Horst, the Netherlands) similar in weight were housed individually in a room with controlled temperature (20–24°C), relative humidity (55% ± 15%) and a 12 h light-dark cycle. Mice were fed the diet and demineralized water ad libitum. In a previous experiment, the concentration of 0.5 µmol heme/g diet was used (12). In a pilot experiment, we studied whether a lower dose of heme (0.2 µmol heme/g diet) has similar effects as observed before on heme-induced hyperproliferation. Thereto, mice received a Westernized purified control diet (0.2 µmol heme/g diet) has similar effects as observed before on heme-induced hyperproliferation. Thereto, mice received a Westernized purified control diet (0.2 µmol heme/g diet) has similar effects as observed before on heme-induced hyperproliferation. Thereto, mice received a Westernized purified control diet (13), or the diet supplemented with either 0.2 or 0.5 µmol heme/g diet (Sigma–Aldrich, St Louis, MO) for 14 days (n = 8 mice per group).

To study the time-dependent effects of heme on the colonic epithelium, mice received either the Westernized control diet (13) or the diet supplemented with 0.2 µmol heme/g. Before the start of the intervention, all mice received the control diet for 1 week. Thereafter, mice received the control or the heme diet for 2, 4, 7 or 14 days (n = 4, per time point, per diet). One control mouse died at day 2 and this group thus contained three mice. Twenty-four hour feces were quantitatively collected at days 2, 4, 7 and 14 of the experiment, frozen at −20°C and subsequently freeze-dried. Mice were anesthetized with isoflurane (1.5% in 70% nitrous oxide and 30% oxygen). After killing, the colon was excised, mesenteric fat was removed and the colon was opened longitudinally. The colon was washed in phosphate-buffered saline and cut into three parts. The middle 1.5 cm tissue was formalin-fixed and paraffin embedded for histology. The remaining proximal and distal parts were scraped. Scraped mucosa was pooled per mouse, snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Fecal analyses

Fecal water was prepared and the cytotoxicity of this fecal water was measured as described previously (12). To determine lipid peroxidation products in the lumen, thiorbarbituric acid reactive substances (TBARS) in fecal water were quantified, according to Okikawa et al. (14). Briefly, fecal water was diluted 4-fold with double-distilled water. To 100 µl of this dilution, 100 µl

Abbreviations: CHF, cytotoxic heme factor; Ihh, Indian Hedgehog; IL–15, interleukin-15; ROS, reactive oxygen species; PPAR, peroxisome proliferator-activated receptor; SEM, standard error of the mean; TBARS, thiorbarbituric acid reactive substances; Vml, vanin-1.
Heme-induced cytotoxic stress initiates hyperproliferation

Immunohistochemistry
Paraffin-embedded colon sections of 5 μm were deparaffinized. Hematoxylin and eosin staining was performed to assess the morphology of the tissue. To stain proliferating cells, sections were stained with an antimouse Ki67 antibody as described previously (12). Colonocytes from 15 well-oriented crypts (longitudinal sections) were counted for each animal. A cell was scored Ki67-positive when the nucleus of the cell was distinctly brown. The number of Ki67-positive cells per crypt, the total number of cells per crypt and the labeling index (% of Ki67-positive cells per crypt) were determined. For Ki67 quantification, only colons of time point 0 and 14 days (n = 4 per time point) were included for the control mice. Regarding the heme-fed mice, all animals from all time points (days 2, 4, 7 and 14; n = 4 per time point) were included.

RNA isolation
Total RNA was isolated by using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer’s protocol. For microarray hybridization, the isolated RNA was further column purified (SV total RNA isolation system; Promega, Leiden, the Netherlands). RNA concentration was measured on a nanodrop ND-1000 ultraviolet–visible spectrophotometer (Isogen, Nieuwegein, the Netherlands) and analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips, according to the supplier’s protocol. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits and displayed no chromosomal peaks or RNA degradation products (RNA integrity number > 8.0).

Array hybridization and microarray data analysis
For the pilot study using different heme concentrations, RNA was isolated from scrapings of three groups receiving 0.2, 0.5 or 0.881 mol heme/g diet (n = 8 mice per group) and pooled per group. To determine the optimal heme concentration, RNA of colon scrapings was isolated and pooled per time point and treatment group. Nine arrays were performed; one at time point 0, two for all other time points. RNA from both studies was analyzed using Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA). Labeling was performed with ‘Affymetrix Whole Transcript Sense Labeling without tRNA reduction step’ according to the WT Sense Target Labeling Assay Manual (Affymetrix). Scanned microarrays were analyzed as described previously (15). Arrays were normalized using the Robust Multiarray Average method (16,17). Probe sets were defined according to Dai et al. (18). Only genes with more than 10 probes on the array and genes with a signal intensity >20 at least in one of the arrays were included in the analysis. A fold-change of 1.3 was set as cut-off for differential regulation. Array data have been submitted to the Gene Expression Omnibus, accession number GSE40671.

Statistical analysis
Physiological data are presented as mean ± standard error of the mean (SEM). Differences in weight and proliferating cells between the different heme concentrations were tested by a one-way analysis of variance with a Bonferroni’s multiple comparison test. For the time-course experiment, differences between heme and control at a certain time point were tested for statistical significance by a two-tailed Student’s t-test. P-values < 0.05 were considered to be significant.

Results
Both 0.2 and 0.5 μmol heme per gram food induce hyperproliferation
We previously showed that dietary heme induced hyperproliferation in mouse colon by downregulating feedback inhibitors of proliferation (12). In that study, using a heme concentration of 0.5 μmol/g diet, body weight was decreased. To prevent weight loss of mice, we wanted to use a lower concentration of dietary heme. Moreover, we hypothesized that for studying the heme-induced effects in time, a low nal concentration of heme is preferred to separate possible differential time-dependent effects. Thereto, we first tested the effects of the more physiological concentration of 0.2 μmol heme/g on proliferation and gene expression at day 14 and compared these effects to the effects of 0.5 μmol heme/g.

After 2 weeks of feeding, mice fed the 0.2 μmol heme/g diet had a similar weight compared with the controls (Table 1). Mice on the 0.5 μmol heme/g diet, however, had a significantly lower body weight after 14 days of intervention (Table 1). Colonic proliferation was investigated by Ki67 staining for n = 4 mice per group. The number of proliferating cells and the total number of cells were quantified and presented in Table 1. Both 0.2 and 0.5 μmol heme/g diets significantly induced hyperproliferation. The total number of cells per crypt and the number of Ki67-positive cells per crypt were significantly higher in the 0.5 compared with the 0.2 μmol heme/g diet, but the labeling index, which is the percentage of proliferating cells per crypt, was similar in both heme-fed groups.

Mucosal gene expression levels were investigated by microarray on pooled scrapings for the control, the 0.2 and the 0.5 μmol heme/g diets. To determine whether mucosal changes were reproducible between studies, changes in gene expression on the 0.5 μmol heme/g diet were correlated to the fold-changes for significant changed genes (n = 3663, q < 0.01) found in our previous identical designed mice study with individual arrays (n = 7–9 mice per group) (12). This yielded a Pearson correlation coefficient of 0.832 (P < 0.000, data not shown), implying that gene expression changes were highly similar and reproducible between both studies. When comparing the expression levels of these 3663 genes within this experiment, comparing 0.2 and 0.5 μmol heme/g, a Pearson correlation coefficient of 0.881 was obtained (P < 0.000, data not shown). This implies that both 0.2 and 0.5 μmol heme/g modulate the expression of similar genes to a similar extent.

Differential time course of heme-induced oxidative and cytotoxic stress in colonic lumen
Next, a time-course study was performed to investigate the causality of heme-induced changes. In this study, mice received a heme diet for 2, 4, 7 or 14 days (n = 4 per time point). For this study, the concentration of 0.2 μmol heme/g diet was used as it increased cell proliferation, without affecting body weight, and induced similar gene expression changes as 0.5 μmol heme/g, described in previous section. Before the start of the experiment, all mice received the control diet for 1 week. Also, in this study, body weight was not significantly different in the heme group compared with the control throughout the experiment (final weights [mean ± SEM]: 26.6 ± 1.0 versus 28.1 ± 1.2 g for heme and control, respectively). The overall morphology of the colonic tissue was visualized by an hematoxylin and eosin staining (Figure 1). There were no signs of inflammation, as there was no infiltration of neutrophils or macrophages in the lamina propria.

Heme induced oxidative stress in the colonic lumen of mice and rats by generating ROS, reflected by a heme-dependent increase

| Table 1. Effects of different heme concentrations on physiological parameters |
|-------------------------------|-----------------|-----------------|
|                              | Control         | 0.2 μmol heme/g | 0.5 μmol heme/g |
| Weight (g)                   | 25.9 ± 0.7*     | 26.0 ± 0.7*     | 20.5 ± 0.6**    |
| Total number of cells per crypt | 40.6 ± 2.1*   | 58.1 ± 2.8**    | 80.1 ± 1.6***   |
| Number of Ki67-positive cells per crypt | 14.3 ± 1.4* | 35.7 ± 3.6** | 46.8 ± 2.2*** |
| Labeling index (%)           | 34.9 ± 1.6*     | 60.7 ± 4.3**    | 57.5 ± 2.0**    |

Data are represented as mean ± SEM, (n = 8 for weight, n = 4 for other parameters) after 14 days of intervention.

*Statistically significant (P < 0.05) results are indicated with (*, ** or ***)

and were tested by one-way analysis of variance with a Bonferroni’s multiple comparison test.
in fecal TBARS (10). TBARS were measured in fecal waters at several time points included in the study. TBARS were significantly higher in the heme-fed mice compared with the controls at day 2 of heme feeding and stayed higher than controls throughout the study (Figure 2A). This implies that dietary heme induced ROS production acutely, that is, within the first 2 days of heme exposure. Besides ROS production, we previously found that dietary heme also induces cytotoxicity of the colonic contents (12). This increase in cytotoxicity is caused by the covalently modified heme metabolites of which the exact structure is unknown (13). In our time-course study, cytotoxicity of fecal water was low in control animals and was stable over time (Figure 2B). Remarkably, there was a lag time in the induction of cytotoxicity of the colonic contents of the heme-fed mice. Cytotoxicity increased after day 4 and was significantly higher on heme compared with the controls on days 7 and 14. The Ki67 staining for proliferation (Figure 1) revealed that heme mice increased their proliferation of colonic epithelial cells from day 4, and the difference was significant on days 7 and 14 (Figure 2C). Concurrently with the number of Ki67 positive cells (Figure 2C) also the labeling index and the total number of cells per crypt were increased (data not shown). Together, these physiological changes show that oxidative stress is an acute heme-induced effect, whereas both cytotoxicity and hyperproliferation are delayed effects.

**Time course of differential heme-modulated transcription factors and processes in colon mucosa**

To get more insight into the heme-modulated cellular processes in time, gene expression patterns were investigated by using whole-genome microarrays. Based on the physiological changes described above, there were two main effects in time; acute effects from day 2 that persisted till day 14 and delayed effects that were initiated after day 4 and lasted till day 14. Ingenuity software (Ingenuity® Systems, www.ingenuity.com) was used to get more insight into the processes and transcription factors, which were involved in these heme-induced acute or delayed effects. Thereto, differentially expressed genes (fold-change >1.3 or < −1.3) for each time point were uploaded and analyzed. Ingenuity showed that the majority of the differentially expressed genes at day 2 played a role in lipid metabolism (Figure 3A). The genes changed after day 4, however, were related to neoplasia/cancer and proliferation. Based on differentially expressed genes, Ingenuity indicates which transcription factors are involved in the induction or repression of these genes. For day 2, these were PPARα and PPARγ, two important transcription factors in lipid metabolism, which were activated (Figure 3B). After day 4, the heme diet activated several oncogenes, such as Myc, Tbx2, Foxm1 and Jun, but inhibited the tumor suppressor TP53 (Figure 3B). Thus, this Ingenuity analysis indicates acute activation of PPARα and PPARγ targets and lipid-related processes, but a delayed induction of tumor-related processes, which is line with the physiological findings described above.

**Heme acutely changed the expression of lipid- and oxidative stress–related genes**

Gene expression levels were determined to see which genes contributed to the acute or delayed heme-induced effects (Figure 4). In line with the Ingenuity results, many of the genes that were up or downregulated at day 2 were involved in fatty acid metabolism according to their GO annotations. Of all the genes changed at day 2, 64% were target genes of PPARs (Figure 4A), which are generally known regulators of lipid metabolism. For the colon, several PPARγ targets were identified (19), but these PPARγ target genes show a large overlap with the PPARα targets (20). Therefore, in Figure 4, expression levels of defined PPARα targets were shown (20). Dietary heme catalyzed the production of ROS, which results in the production of oxidized lipids. These oxidized lipids are ligands for PPARs, and most probably induced the expression of PPAR target genes on the heme diet. Besides PPARα targets, 11% of differentially expressed genes were upregulated Nrf2-target genes, such as catalase. Nrf2 was also present in the Ingenuity results as involved transcription factor at day 2 with a P-value of 0.0013. However, Nrf2 had a z-score <2 and was therefore not presented in Figure 3B. Vanin-1 (Vnn1), which is a PPARα target and a marker of oxidative stress (21), was also acutely upregulated (Figure 4A).

**Delayed mucosal sensing of heme and cytotoxicity coincided with hyperproliferation**

Gene expression levels for the delayed effects are shown in Figure 4B. In total, there were more delayed than acute differentially expressed genes (381 versus 44), and the delayed...
Heme-induced cytotoxic stress initiates hyperproliferation

As luminal cytotoxicity was increased, after day 4, we determined when the increased cytotoxicity affected the epithelial surface cells. Therefore, the expression of cytotoxic-stress and cell-death-related genes was investigated (Figure 4B). In our previous study, we showed that dietary heme induced surface-specific inhibition of cap-dependent protein translation by increasing epithelial cell levels of the endoplasmic reticulum stress marker Eif4ebp1 (4E-BP1) (12). In this time-course study, we found that 4E-BP1 was upregulated after day 4, which coincides with the increased cytotoxic stress. The induced expression of glutathione metabolism–related genes between days 4 and 7 indicated an increased need for protection against noxious compounds. With regard to cell death, we found that the apoptosis inhibitor survivin (Birc5) was upregulated at day 4. Recently, we determined that this is a crypt-specific upregulation (12). Another apoptosis inhibitor, immediate early response 3 (Ier3) was also upregulated after day 4 and this is a surface cell–specific upregulation (12). Besides apoptosis inhibitors, the necrosis inducer receptor interacting protein kinase-3 was significantly upregulated upon heme feeding after day 4. This indicated that after 4 days of heme feeding, there was a shift in type of cell death from apoptosis to luminal necrosis, which is in line with our earlier studies (12,26).

As the mucosa sensed cytotoxicity after day 4, signals from the damaged surface cells had to trigger the transit-amplifying crypt cells for compensatory hyperproliferation. We showed recently that dietary heme initiated this hyperproliferation, resulting in hyperplasia, by downregulating feedback inhibitors of proliferation, such as Wnt inhibitory factor 1, Ihh, bone morphogenetic protein 2 and IL-15, in the surface epithelium (12). When investigating gene expression changes of these genes in time, we found a downregulation of Wnt inhibitory factor 1, bone morphogenetic protein 2 and IL-15 after day 4, whereas Ihh remained constant (Figure 4B). The downregulation of these three signaling molecules coincided with the increase in cell proliferation (Figure 2C) and with the upregulation of cell cycle–related genes (Figure 4B). The Ki67 gene expression levels were significantly higher in heme-fed mice compared with controls after day 4, which is in line with their protein levels (Figure 1). Also in line with increased cell cycling, Plk1, Mad2l1 and Cdc20, belonging to the Kyoto Encyclopedia of Genes and Genomes (KEGG) cell cycle pathway, were upregulated after day 4 (Figure 4B). Moreover, cyclins A2 and B2 were significantly higher expressed in heme-fed mice compared to controls after day 4.

Taken together, these gene expression results show that the mucosal response to luminal oxidative and cytotoxic stress coincided with the differential time course of the luminal stressors. This is illustrated for selected marker genes in Figure 5A. Catalase expression showed that oxidative stress was sensed early on. However, sensing of cytotoxic stress (e.g. receptor interacting protein kinase-3) occurred after day 4. This coincided with the downregulation of the surface to crypt signaling proliferation inhibitors (e.g. IL-15) and upregulation of cell turnover genes (e.g. Ki67). Remarkably, there was also delayed heme sensing (Hmox1). This raised the question why heme was sensed only after day 4, whereas heme is present in the colon at day 1, as the oral fecal transit time in rodents is much <24 h. This implies that there was a barrier between the heme present in the luminal contents and the surface epithelial cells. The continuous mucus layer in the colon protects the surface cells against the luminal heme compounds. We investigated whether there were time-dependent changes in the expression of mucin and O-glycosylation genes. Only the expression of the cell surface mucin 1 and the core-2 glucosaminyl (N-acetyl) transferase 1 was downregulated after day 4 (Figure 5A). Analysis of the expression database from our recent laser capture microdissection study (12) showed that the heme-induced downregulation of these genes occurs exclusively in the surface epithelium (Figure 5B). This indicates that there might be a reduced mucin barrier function after day 4 on the heme diet.

**Discussion**

To our knowledge, this is the first study showing that the effect of dietary heme on the colon epithelium can be separated into an acute.

**Fig. 2.** The effects of the heme diet on TBARS (A), cytotoxicity (B) and proliferation in time (C). TBARS and cytotoxicity measurements were performed in triplo. Ki67 was evaluated for all heme-fed mice and for controls on days 0 and 14. Data are represented as mean ± SEM (n = 4). *indicates significant difference with P < 0.05.
ROS stress and a delayed cytotoxic stress. The increased ROS production, which was present within the first 2 days of heme feeding, induced the formation of lipid peroxidation products. These lipid peroxidation products are ligands for PPARs (27–29) and may thus explain the acute activation of PPAR target genes. Besides, ROS can activate Nrf2, which is a transcription factor regulating the antioxidant response via the antioxidant response element–mediated induction of many cytoprotective enzymes, such as catalase. Several Nrf2 targets were induced at day 2 after heme consumption and stayed higher expressed throughout the experiment. Vnn1 is an oxidative stress marker (21), which was upregulated from day 2. Besides an oxidative stress marker, Vnn1 is recently proposed as a causal factor in colonic hyperproliferation (30). However, this is not corroborated by our study, as no increase in proliferation was observed concomitant with Vnn1 upregulation. Together, these gene expression changes and the changes in TBARS show that shortly after heme consumption increased oxidative stress is present. However, there were no early changes in cell cycle genes observed (up to day 4), indicating that luminal ROS does not directly cause hyperproliferation.

After day 4, the expression of several cytotoxic stress markers was upregulated, which coincided with the induction in luminal cytotoxicity. We have shown before that the immediate early response gene Tis7 is upregulated by dietary heme especially at the surface epithelium (12). In this study, we show that Tis7 was upregulated after day 4, indicating a delayed effect. The induction of the necrosis inducer receptor interacting protein kinase-3 and the stress-related gene Nemo was also delayed. From our previous study, we also know that protein translation is inhibited in heme-fed mice specifically at the surface epithelium, shown by an upregulation of the translation inhibitor 4E-BP1 (12). Here, we show that 4E-BP1 was upregulated after day 4 and thus coincided with the increased cytotoxic stress. It is most probable that cytotoxicity damages the surface cells, which subsequently inhibit their protein translation to prevent energy dissipation. The induction of 4E-BP1 inhibits protein translation, which implies that protein levels of the identified signaling molecules involved in proliferation are lower than would be expected based on gene expression changes. Although there were no significant changes found at gene expression level for the signaling molecule Ihh, Ihh protein levels can actually be lower under heme conditions due to the translation inhibition by 4E-BP1.

Besides the increased cytotoxicity, hyperproliferation and hyperplasia are also induced by heme after day 4 both at gene expression level as well as for the Ki67 counts. In line with this, there was activation of several oncogenes and inhibition of the tumor suppressor TP53. Earlier dietary interventions with bile acids (31) or calcium (32) showed that luminal cytotoxicity is causal for hyperproliferation. This study supports this causality as both the heme-induced cytotoxicity and hyperproliferation originate simultaneously. The delayed luminal cytotoxicity is in line with our proposed mechanism for the formation of the cytotoxic heme factor (CHF). As described earlier (11), we believe that CHF is formed in the colonic lumen via covalent addition of reactive lipid peroxides to the protoporphyrin ring of heme, resulting in very lipophilic products with a molecular weight higher than that of heme (13). This implies that lipid peroxides, measured as TBARS, must first accumulate in the colonic lumen before substantial CHF formation can occur. With the low heme concentration in this study, this apparently requires about...
Heme-induced cytotoxic stress initiates hyperproliferation. This lag time is dependent on the heme dosage and should be shorter with higher heme intake. Indeed, an earlier pilot experiment in our laboratory showed that cytotoxicity was already increased at the earliest time point, that is, day 3, using a diet with 0.6 µmol heme/g in rats (Sesink and van der Meer, unpublished observation).

Unfortunately, final proof for this mechanism is lacking as the precise structure of CHF remains unknown. We could not obtain its mass spectrum in different types of mass spectrometers because CHF could not be ionized, which is a known problem with complex lipophilic compounds. However, there are several dietary interventions, which support our mechanism. For instance, heme-induced TBARS and cytotoxicity are almost completely prevented by antioxidants (33), replacement of polyunsaturated fat by monounsaturated fat (33), and by the heme antagonist chlorophyll, mimicking intake of vegetables (13,34). It should be noted that these interventions also concomitantly prevented heme-induced hyperproliferation (13,34) and aberrant crypt formation (33), illustrating the causal role of luminal cytotoxicity. Taken together, these studies show that formation of CHF in the colonic lumen is critically dependent on the presence of lipid peroxides. Thus, although lipid peroxides themselves do not cause hyperproliferation, they are indirectly causal via the formation of CHF.

We speculate that this heme → CHF → cytotoxicity → hyperproliferation sequence in colon may also be modulated by other luminal factors such as the microbiota. This is because bacteria are able to catabolize heme (35) and other porphyrins (36) and thus may interfere with the formation of CHF. In addition, the colon microbiota metabolizes the protective mucus layer and modulates the expression of the genes controlling the mucin barrier (37). With regard to this, it may be of significance that the downregulation of mucin 1 and glucosaminyl (N-acetyl) transferase 1 (Figure 5B) coincides with the increase in heme-induced early and delayed response genes (fold change > 1.3 or fold change < −1.3) at day 2, of which many were involved in lipid metabolism and oxidative stress based on KEGG pathways. Red indicates upregulation and green indicates downregulation. Color scale ranges from a signal log ratio of −2 (green) to 2 (red). Panel B contains delayed heme-induced genes. The day on which these genes were differentially expressed (fold change > 1.3 or fold change < −1.3) is indicated in the last column. Color scale ranges from a signal log ratio of −1.5 (green) to 1.5 (red).
in cytotoxicity. This is not due to a generic cytotoxic effect, as the expression of other mucin genes was not changed. Therefore, this may indicate a specific change in luminal signals reflecting heme-induced changes in microbiota composition (38,39).

In this study, we used heme as a proxy for the consumption of red meat. The results show that a low concentration of heme (0.2 µmol heme/g) already induces cytotoxicity and epithelial hyperproliferation. This is in line with earlier studies in rats showing that 0.16 and 0.25 µmol heme/g induce cytotoxicity leading to hyperproliferation (40) and aberrant crypt formation (33), respectively. As an average human diet consists of about 400 g dry weight/day, 0.2 µmol heme/g diet corresponds to 80 µmol heme/day. As beef contains 0.5 µmol heme/g wet weight (41), this implies that 0.2 µmol heme/g used in this study mimics a daily intake of 160 g red meat, which is similar to the high intake of red meat in Westernized countries, observed in epidemiological studies (4,5,42). As lifelong intake of meat often occurs in humans, the question arises whether our heme-induced changes in luminal cytotoxicity and mucosal cell turnover are maintained during longer treatments. This is indeed the case as we had found similar changes after 14 weeks on a heme diet (33). Therefore, it would be worthwhile to perform an even longer study to determine whether heme induces spontaneous tumor formation in colon mucosa.

In conclusion, dietary heme causes an acute luminal ROS production and oxidative stress response in the mucosa. Delayed, dietary heme increases the cytotoxicity of the colonic contents and simultaneously heme-induced hyperproliferation is observed. Therefore, heme-induced hyperproliferation is related to cytotoxic stress rather than oxidative stress. Whether changes in colon microbiota play a causal role in the delayed cytotoxicity and hyperproliferation will be investigated further by using antibiotics.

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**References**

Heme-induced cytotoxic stress initiates hyperproliferation


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