Gut microbiota facilitates dietary heme-induced epithelial hyperproliferation by opening the mucus barrier in colon

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Colorectal cancer risk is associated with diets high in red meat. Heme, the pigment of red meat, induces cytotoxicity of colonic contents and elicits epithelial damage and compensatory hyperproliferation leading to hyperplasia. Here we explore the possible causal role of the gut microbiota in heme-induced hyperproliferation. To this end, mice were fed a purified control or heme diet (0.5 μmol/g heme) with or without broad-spectrum antibiotics for 14 days. Heme-induced hyperproliferation was shown to depend on the presence of the gut microbiota, since hyperproliferation was completely eliminated by antibiotics, although heme-induced luminal cytotoxicity was sustained in these mice. Colon mucosa transcriptomics revealed that antibiotics block heme-induced differential expression of oncogenes, tumor-suppressors and cell-turnover genes, implying that antibiotic treatment prevented the heme-dependent cytotoxic micelles to reach the epithelium. Our results indicate that this occurs because antibiotics reinforce the mucus barrier by eliminating sulfide-producing bacteria and mucin-degrading bacteria (e.g. *Akkermansia)*. Sulfide potently reduces disulfide bonds and can drive mucin denaturation and microbial access to the mucus layer. This reduction results in formation of trisulfides that can be detected *in vitro* and *in vivo*. Therefore, trisulfides can serve as a novel marker of colonic mucolysis and thus as a proxy for mucus barrier reduction. In feces, antibiotics drastically decreased trisulfides, but increased mucin polymers that can be lysed by sulfide. We conclude that the gut microbiota is required for heme-induced epithelial hyperproliferation and hyperplasia by their capacity to reduce mucus barrier function.

colorectal cancer | red meat | mucus barrier | mucolysis | (tri)sulfides

Introduction

Colorectal cancer, the second leading cause of cancer death in Western countries, is associated with diets high in red meat (1), whereas consumption of white meat does not have this association (2). Heme, the iron-porphyrin pigment, is present at much higher levels in red- compared to white meat. Epidemiological studies show that heme intake is related to colon cancer risk (3, 4). Our previous studies show that when rodents consume heme, their colonic contents become more cytotoxic (5, 6). This increased cytotoxicity injures the colonic epithelial surface cells. To replace the injured surface cells, hyperproliferation from the stem cells in the crypts is initiated. Together with inhibition of apoptosis, this compensatory hyperproliferation leads to hyperplasia (6), which eventually can develop into colorectal cancer.

Dietary heme is poorly absorbed in the small intestine; approximately 90% of dietary heme enters the colon (7). Besides the toxic effect of heme on the colonic mucosa, dietary heme affects the microbiota. The relationship between intestinal microbiota and colon cancer has long been suspected (8). In humans, a red-meat diet increases *Bacteroides* spp. in feces (9). We recently showed that in mice, a heme diet changed the microbiota drastically, majorly increasing the Gram-negative bacteria (mainly Bacteroidetes, Proteobacteria and Verrucomicrobia) (10). The gut microbiota can induce hyperproliferation via mechanisms occurring in the colon lumen, such as modulation of oxidative and cytotoxic stress or by influencing the mucus barrier. Oxidative stress induces the formation of peroxidized lipids, which react with heme to form the cytotoxic heme factor (CHF), thereby increasing cytotoxic stress (5, 11). In a time-course study we showed that there is a lag time in the formation of CHF and in the induction of hyperproliferation when mice are transferred from a control to heme diet (11). This could be due to a time-dependent adaptation of the microbiota to the heme diet. Notably, heme does not increase cytotoxicity and epithelial hyperproliferation in the small intestine (12), indicating that formation of CHF only occurs in the colon where bacterial density is high. Moreover, these experiments suggested that CHF-induced hyperproliferation coincided with a reduced mucus barrier function (11), leading to enhanced contact of colonocytes with microbiota and toxic substances. In the present study we investigate whether bacteria play a causal role in heme-induced cytotoxicity and hyperproliferation by using broad-spectrum antibiotics (Abx). Our results

Significance

Consumption of red meat is associated with increased colorectal cancer risk. We show that the gut microbiota is pivotal in this increased risk. Mice receiving a diet with heme, a proxy for red meat, show a damaged gut epithelium and a compensatory hyperproliferation that can lead to colon cancer. Mice receiving heme together with antibiotics do not show this damage and hyperproliferation. Our data indicate that microbial hydrogen sulfide opens the protective mucus barrier and exposes the epithelium to cytotoxic heme. Antibiotics block microbial sulfide production and thereby maintain the mucus barrier that prevents heme-induced hyperproliferation. Our study indicate fecal trisulfide as a novel biomarker of mucus barrier integrity, which could be of relevance in human colon disease diagnostics.

Reserved for Publication Footnotes

Table 1. Effects of heme and Abx on body weight and fecal parameters

	Control	Heme	Control + Abx	Heme + Abx
Body weight (g)	27.7 ± 0.5 [a]	24.9 ± 0.5 [b]	27.2 ± 0.3 [a]	24.8 ± 0.4 [b]
Fecal wet weight (g/day)	0.49 ± 0.08 [a]	0.60 ± 0.05 [a]	0.62 ± 0.09 [a]	1.20 ± 0.19 [b]
Fecal dry weight (g/day)	0.12 ± 0.01 [a]	0.11 ± 0.01 [a]	0.12 ± 0.01 [a]	0.19 ± 0.02 [b]
TBARS (MDA equivalents, µmol/L)	12.70 ± 1.43 [a]	59.84 ± 2.46 [b]	11.06 ± 1.63 [a]	46.06 ± 3.92 [c]
Cytotoxicity (% lysis)	1.09 ± 0.45 [a]	66.90 ± 10.45 [b]	0.05 ± 0.04 [a]	31.30 ± 8.98 [c]

Mean ± SEM (n=9 per group), differences were tested by ANOVA and Bonferroni post hoc-test. Superscripts indicate significant differences (p<0.05).

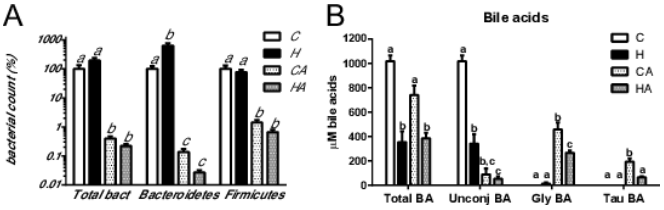

Fig. 1. A. Counts of total bacteria, Bacteroidetes and Firmicutes measured by qPCR. Control bars are set at 100%, other bars are relative to controls; mean ± SEM (n=9/group). B. Bile acid (BA) profiles determined by HPLC; mean ± SEM (n=3/group). Letters indicate significant different groups (p<0.05), ANOVA with Bonferroni post-hoc test. (Gly; glycine conjugated, Tau; taurine conjugated, Sec; secondary

illustrate the crucial role of the gut microbiota in heme-induced hyperproliferation, and indicate the involvement of microbial hydrogen sulfide formation in reduction of the polymeric mucin network to degrade the protective mucus barrier and expose the colon mucosa to CHF.

Results

Heme- and Abx-induced changes in the colonic lumen. Mice were divided into 4 groups receiving either a control diet (further referred to as C-group), a heme diet (H-group), a control diet with Abx treatment (CA-group), or a heme diet with Abx treatment (HA-group). After 2 weeks of intervention the H- and HA-group had a lower body weight as compared to their controls (Table 1). Fecal dry and wet weight was significantly increased in the HA-group (Table 1).

To confirm that Abx decreased the abundance of bacteria, qPCR analyses with specific primers targeting total bacteria, Bacteroidetes or Firmicutes were performed (Fig. 1A). Abx treatment significantly reduced the abundance of total bacteria and Firmicutes ~100-fold and Bacteroidetes ~1,000-fold. Moreover, the H-group had a significantly increased abundance of Bacteroidetes compared to the C-group, which corroborates previous observations (10). Abx thus drastically decreased the bacterial density, affecting both the Gram-positive Firmicutes and Gram-negative Bacteroidetes. To study how this impacts the normal microbial modification of host compounds, we determined the fecal bile acid composition. No conjugated bile acids were detected in fecal water in the C- and H-group, where unconjugated and secondary bile acids were predominant (Fig. 1B). However, with Abx almost all bile acids were primary and conjugated with glycine or taurine (ratio about 3), showing that Abx blocked microbial bile deconjugation and dehydroxylation almost completely.

Heme increases oxidative and cytotoxic stress in the colon (10, 11). Reactive oxygen species (ROS) induce the formation of lipid peroxides which react with heme to form CHF, thereby increasing the cytotoxicity of luminal contents (5, 11). We determined lipid peroxidation product levels by measuring TBARS in fecal water. TBARS were low in the C- and CA-group (Table 1) and increased significantly and to a similar extent in the H- and HA-group, implying that heme, both in presence and absence of Abx, induced ROS-stress. Analogously, fecal water cytotoxicity (Table 1) was significantly increased in the H- and HA-group compared to their controls. Since Abx drastically reduced microbiota density (100-1000 fold), but only slightly reduced cytotoxicity (2-fold) and TBARS (1.3-fold), it is unlikely that bacteria play a major role in the formation of TBARS and cytotoxicity.

Heme- and Abx-induced changes in the colonic mucosa. Morphology analyses of H&E stained colon tissue (Fig. 2A) confirmed the previously reported heme-induced increased crypt depth (H- vs C-group). Abx treatment did not affect the colon morphology in the CA- vs C-group, but completely restored tissue morphology in the HA- vs H-group. The crypt depth increase in the H-group did not result from inflammation since neutrophil and macrophage infiltration in the lamina propria was comparable to the C-group. Analogous to earlier reports (6), cell-proliferation quantification using Ki67-staining (Fig. 2B and 2C) shows that the heme diet strongly induced cell proliferation (H- vs C-group), leading to expansion of the proliferative compartment and increased crypt depth. Abx treatment led to slightly reduced numbers of cells per crypt in the CA- vs C-group, but did not significantly affect their labeling index or amount of proliferative cells. However, Abx treatment in the heme diet (HA- vs H-group) completely suppressed heme-induced hyperproliferation and hyperplasia to levels observed in the C- and CA-groups (Fig. 2C). In conclusion, heme-induced hyperproliferation and hyperplasia in mouse colon only occurs in the presence of the gut microbiota.

Abx block the heme-induced expression of cell cycle genes. Using whole genome transcriptomics we investigated whether the physiological changes were reflected in gene expression profiles. The differentially expressed genes in the H- vs C- and HA- vs CA-groups are visualized in Fig. S1A. The heme diet (H- vs C-group) led to 5,507 differentially expressed genes (q-value<0.01), of which almost 90% (4,859) were not significantly affected in the HA vs CA comparison. The 4,859 genes specific for the H-group were analyzed by Gene Set Enrichment Analysis (GSEA), indicating that mainly cell cycle related processes were affected by heme (Fig. S1B). Moreover, mining of these genes for the involved transcription factors (Fig. S1C), revealed that Cdkn2a, Smarcb1, and the tumor suppressors Tp53 and Rb1 were inhibited, while oncogenes such as Myc and Foxm1 as well as cell cycle regulators E2f1 and Tbx2, were activated by heme. Importantly, these processes and transcription factors were not modulated in the HA-group compared to the CA-group. There were only 369 differentially expressed genes unique for the HA-group (Fig. S1A). Notably, none of the modulated processes identified in the HA-group related to the endpoints of our study. Because of the specific heme-Abx interaction, the Abx-mediated differential gene expression profiles and processes were substantially different in the heme diet background (Table S1 and S2) as compared to the control diet background (Table S3 and S4). These observations indicate that heme-induced mucosal gene expression changes of cell cycle related processes require the presence of the microbiota, which is in agreement with the microbiota requirement for the increased labeling index (Fig. 2C).

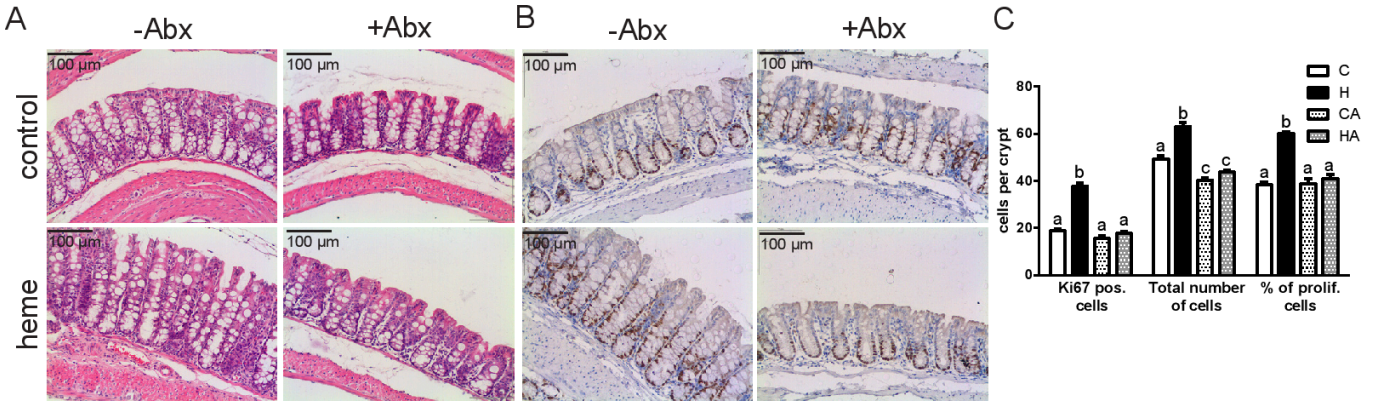

Fig. 2. A. Histochemical H&E staining and B. immunohistochemical Ki67-staining of colon of control and heme-fed mice. C. Quantification of Ki67 positive cells per crypt, total number of cells per crypt and labeling index (percentage of proliferative cells per crypt); mean ± SEM (n=9/group). Letters indicate significant different groups (p<0.05), ANOVA with Bonferroni post-hoc test.

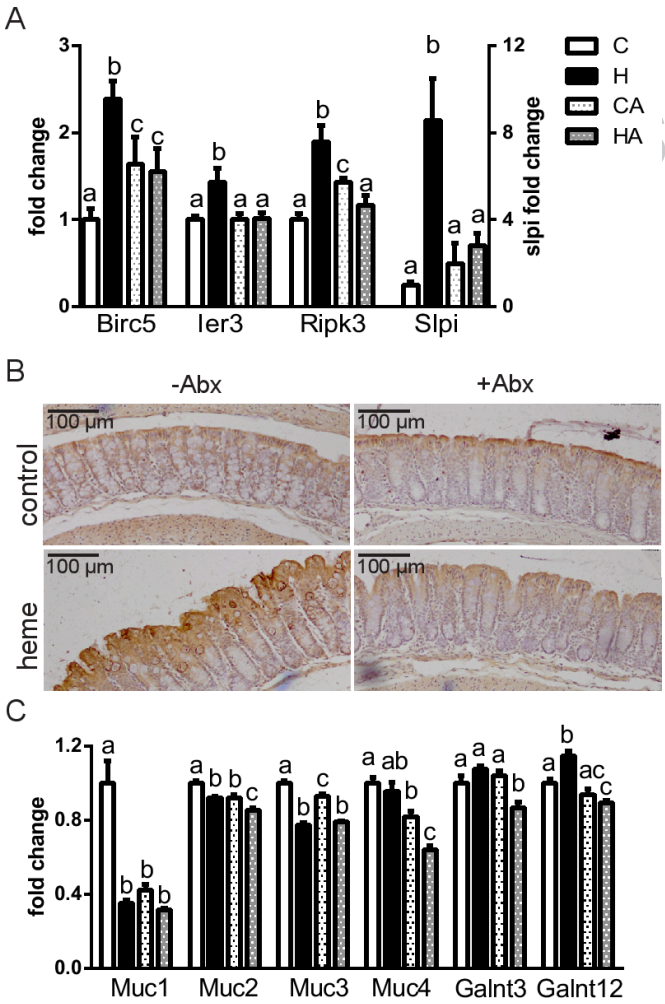

Fig. 3. A. Gene expression of injury markers Birc5, Ier3, Ripk3 and Slpi. B. immunohistochemical colonic Slpi-staining of control and heme-fed mice. C. Gene expression of mucin genes 1 to 4 and Galnt 3 and 12. Expression levels of control is set at 1. Expression of other bars is relative to controls; mean ± SEM (n=4 for C, H, CA; n=6 for HA). Letters indicate significant differences (p<0.05), ANOVA with Bonferroni post-hoc test.

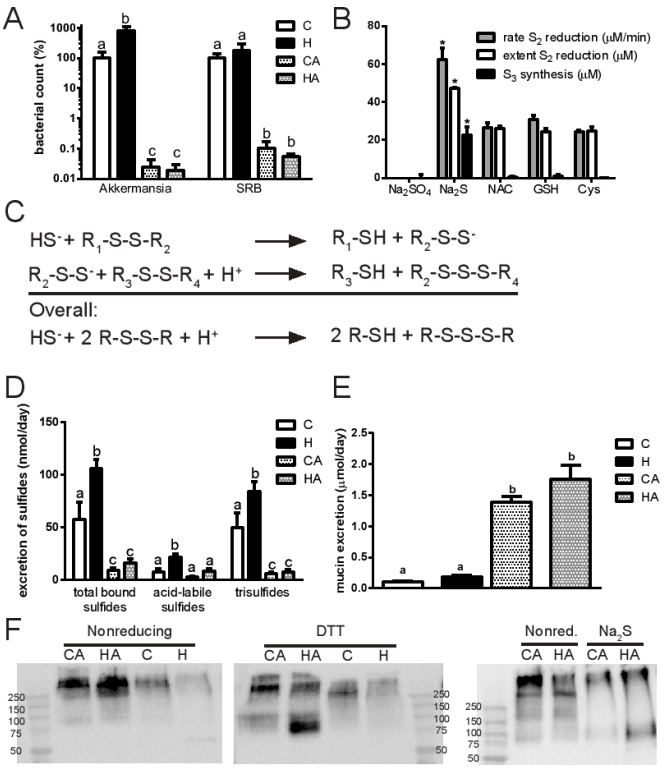

Fig. 4. A. Bacterial counts of *Akkermansia muciniphila* and sulfate reducing bacteria (SRB) determined by qPCR. Controls are set at 100% and other bars are relative to controls; mean ± SEM (n=8-9/group). Letters indicate significant different groups (p<0.05). B. Rate and extent of S-S bond splitting and synthesis of trisulfide bonds; mean ± SD (n=3-6/group),* indicates significant difference with thiol groups (p<0.05). C. Reaction scheme by which sulfide splits S-S bonds. D. Concentrations of sulfides in fecal water; mean ± SEM (n=8-9/group). E. Excretion of fecal mucins, expressed as µmol O-glycan per day (n=6-9/group). F. Western blot analysis of fecal mucin with or without DTT as reducing agent and with sulfide. Samples were stained with anti-Muc2 antibody. Each lane represents a pool of n=9/group. Letters indicate significant different groups (p<0.05), ANOVA with Bonferroni post-hoc test.

Abx do not affect the heme-induced antioxidant response. A set of 648 genes were significantly regulated in both the H- and HA-group as compared to their controls (Fig. S1A). Of those shared genes, 599 were similarly regulated in both groups. Notably, this group of genes included the activation of several transcription factors, including the PPARs, involved in fatty acid metabolism, and Nrf2, involved in antioxidant response (Fig. S1B and C). This implies that oxidative stress and lipid peroxidation products induced the antioxidant response and the induction of

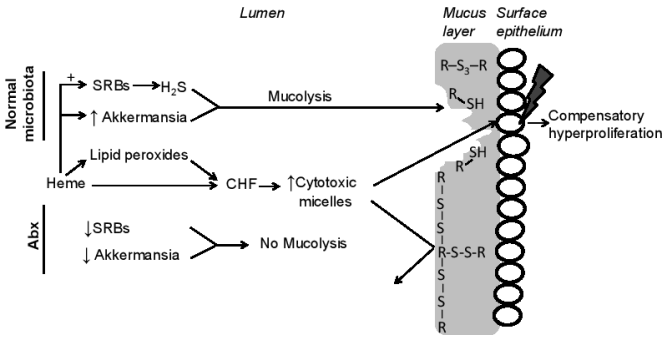

Fig. 5. Proposed mechanism of how microbiota facilitates heme-induced compensatory hyperproliferation. Upper part represents processes when normal microbiota is present (i.e. without Abx) leading to compensatory hyperproliferation. Lower part illustrates how Abx cause that the mucus layer is protective against cytotoxic micelles. R-S-S-R indicates native intra- and intermolecular disulfide bonds in the mucus that can be reduced by H_2S to thiols (R-S-H) and trisulfides (R-S-S-S-R). SRB; sulfate-reducing bacteria, CHF; cytotoxic heme factor.

PPAR target genes in the mucosa, irrespective of the addition of Abx to the heme diet.

Abx block the mucosal sensing of heme-induced luminal cytotoxicity. In the colon, the mucus preserves the epithelial barrier by protecting the surface epithelial cells through creating a diffusion barrier for toxic micelles (13). In the HA-group, substantial luminal cytotoxicity was present, but did not induce hyperproliferation, indicating that CHF did not reach the surface epithelial cells. We investigated epithelial sensing of cytotoxicity using the marker-genes survivin (Birc5), immediately early response 3 (Ier3), the necrosis facilitator Ripk3, and secretory leukocyte protease inhibitor (Slpi), which are upregulated by cell injury as described earlier (11, 14). In the present study, heme upregulated the expression of these cytotoxicity sensors only in the H-group, but not in the HA-group (Fig 3A). In addition, Fig 3B shows that heme only injured surface cells, which corroborates our laser-capture analysis (6). Taken together, these results indicate that the microbiota is required for the cytotoxic micelles to reach the surface epithelium, as the diffusion barrier of the mucus layer was maintained by Abx.

An increased mucus barrier could be caused by increased mucin synthesis, or by decreased mucin degradation. Expression levels of secreted Muc2 and cell-associated Muc4 were decreased in the HA- compared to the H-group (Fig. 3C). Moreover, the KEGG pathway 'Mucin type O-Glycan biosynthesis' was significantly repressed in the HA- compared to the H-group, according to GSEA analysis (q=0.049). For instance, Galnt 3 and 12, involved in the first step of o-glycosylation, were downregulated by Abx (Fig. 3C), indicating that mucus layer production is most probably decreased by Abx. Regarding mucus degradation, it is well established that the microbiota degrades mucins and uses their carbohydrates and amino acids as substrates for growth. In addition, sulfate-reducing bacteria (SRBs) use mucin-derived sulfate as electron acceptor in anaerobic respiration forming sulfides (see also below). As the Abx-treatment drastically reduced the microbiota density, we hypothesized that Abx increased the mucus barrier by preventing microbial degradation of mucin. Notably, the abundance of the mucin-degrading *Akkermansia muciniphila* was 8-fold increased by heme, whereas Abx reduced the abundance of this mucin degrader more than 1000-fold (Fig. 4A). This implies that the relative reduction of the *Akkermansia* population by Abx exceeded the overall effect of Abx on the total microbial load, supporting that Abx treatment could increase mucus barrier function by strongly reducing the levels of mucin degraders such as *Akkermansia*.

Sulfide reduces S-S bonds leading to mucolysis. Mucus is high in intra- and intermolecular S-S bonds, stabilizing its polymeric, network-like structure. When S-S bonds are broken, mucin monomers dissociate and/or denaturate, leading to decreased viscosity and higher mucus accessibility for bacterial degradation (15). SRBs can use mucus-derived sulfate as oxidant in anaerobic respiration, generating sulfide. We hypothesized that sulfide could have a mucolytic effect by reducing the intermolecular S-S bonds, which contributes to an enhanced mucus barrier in the Abx groups due to the decreased abundance of SRBs (Fig. 4A) and the corresponding decrease of luminal sulfide production. We tested the reducing potency of several sulfur containing compounds on the model compound DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) containing a central S-S bond. Splitting of this S-S bond in DTNB leads to increased absorbance at 412 nm, and the assay allows the determination of the overall S-S splitting extent as well as the initial S-S splitting rate (Fig. 4B). Cysteine, glutathione and N-acetylcysteine (NAC) were able to split the S-S bond with a similar rate between 24 to 31 μM/min, whereas the negative control Na_2SO_4 did not affect DTNB integrity. Importantly, sulfide gave a significantly higher rate of S-S bond splitting, 62.4 ± 5.9 μM/min, indicating that sulfide has a 2-fold more potent mucolytic effect as compared to the amino-acid thiols that have been shown to split S-S bonds and make mucins less viscous (16). Moreover, the overall extent of S-S bond splitting by sulfide was also 2-fold higher as compared to the other amino-acid thiols. Based on Ellman's mechanism (17), this indicates that a reactive persulfide anion originates from the splitting of the first S-S bond, which can subsequently target a second S-S bond, creating a trisulfide bond. To quantitate the production of trisulfide bonds, we developed a method to determine trisulfide bonds based on the difference between total bound sulfides and acid labile sulfides (18). Indeed, trisulfide bonds were generated when sulfide was used to reduce S-S bonds in DTNB (Fig. 4B), but not upon amino-acid thiol (NAC, GSH or cysteine) treatment of DTNB. The theoretical (Fig. 4C) as well as the measured ratio (Fig. 4B) of sulfide-dependent formation of thiol to trisulfide is 2, indicating that all sulfide reacts with DNTB to form trisulfide bonds.

To test whether similar redox reactions also occurred *in vivo*, total sulfides and trisulfides were determined in mice fecal water (Fig 4D). Heme increased the levels of total bound sulfides, which is in agreement with literature showing that heme addition to the growth medium stimulates bacterial reduction of sulfate to sulfide (19). Concentrations of trisulfide in fecal water of mice not receiving Abx were much higher (with H>C) than those of mice receiving Abx, indicating that Abx suppressed sulfide-dependent splitting of S-S bonds and thus colonic mucolysis. In line with this, Abx drastically increased fecal excretion of mucin (Fig. 4E), because the slightly lower steady-state mucin synthesis (Fig. 3C) is not anymore balanced by its bacterial degradation. Fecal mucin was present as a high-molecular-weight form of Muc2 that could not penetrate the gel in SDS/PAGE analysis (Fig. 4F), whereas with DTT as a reducing agent most of the Muc2 appeared as a band of low MW in the gel. To corroborate the reducing effect of sulfide, Muc2 Western blotting was repeated for the CA- and HA-groups and showed that also sulfide lysed the polymeric Muc2 almost completely (Fig. 4F). Overall, these results indicate that in normal colon physiology, microbial sulfide opens the polymeric Muc2 network to bacterial degradation resulting in a lumen-to-surface permeability gradient through the mucus layer. Abx block these microbial processes and thereby increase the mucus barrier.

Discussion

This study shows that dietary heme changes the microbiota and increases ROS and cytotoxicity in the colonic lumen. In addition, heme injures the surface epithelium leading to compensatory hyperproliferation, hyperplasia, and differential expression of

tumor-suppressor and oncogenes, which increases colorectal cancer risk (20, 21). These luminal and epithelial effects of heme are similar to those detailed in our recent studies (6, 10, 11). The crucial finding of the present study is that when the microbial abundance is drastically reduced by Abx, heme does not injure the surface epithelium and does not induce the carcinogenic changes in the crypts, mentioned above. This is not due to the slightly lower levels of cytotoxicity and ROS in the HA-group, which can be explained by the higher luminal dilution factor because of the increased fecal wet weight. Recently, we reported that heme diets induce, in the colon lumen, covalent heme modification resulting in the very lipophilic and toxic CHF, which is solubilized in mixed micelles (11). Abx block the mucosal sensing of these cytotoxic micelles, as they prevent the heme-induced changes in epithelial histology, and in up regulation of injury markers such as Slpi. In contrast, Abx do not block mucosal sensing of luminal ROS, as the Nrf2-mediated antioxidant response was initiated and PPARs were activated by oxidized lipids in both heme diet groups. This differential mucosal sensing shows that, with Abx, the mucus layer is still permeable to small molecules, such as oxidized lipids, but no longer to larger micellar aggregates containing CHF. The absence of hyperproliferation in the HA-group also shows that mucosal exposure to ROS does not cause hyperproliferation. This is in line with our previous observation that ROS is instantly formed after consumption of the heme diet, while there is a delay in the appearance of luminal cytotoxicity and the induction of hyperproliferation (11).

Our study implies that the colon microbiota facilitates heme-induced epithelial injury and hyperproliferation by opening the mucus barrier by the concerted action of hydrogen sulfide-producing and mucin-degrading bacteria. The principal steps of this hypothesis (Fig. 5) are (i) mucolysis by hydrogen sulfide to open the compact, protective mucus layer for (ii) further bacterial degradation, thereby (iii) allowing diffusion of luminal, cytotoxic, micelles to the mucosal surface. Consequently, surface epithelial cells are less protected against luminal cytotoxicity, leading to induction of compensatory hyperproliferation. The diffusion barrier function of the mucus layer is illustrated by Muc2 KO mice, which display colitis and epithelial hyperproliferation, as well as spontaneous development of colorectal cancer (22). In addition, an *in vitro* study shows that apically applied mucin creates a diffusion barrier preventing the contact between cytotoxic micelles and colonocytes (13). That microbiota increase the permeability of mucus barrier is illustrated by a study of recolonized vs Abx-treated rats showing that bacteria colonizing the isolated colonic segment increase epithelial injury by luminally added toxic compounds (23). Unfortunately, there are no established methods to determine the permeability of the mucus layer *in vivo.* Although the mucus layer can be stained in appropriately fixed intestinal samples, this does not provide information about its permeability, as its thickness and permeability are not inversely related (15).

Central to our hypothesis is that hydrogen sulfide can reduce and thus split S-S bonds, which opens the mucus layer. The compactness of this layer is determined by disulfide bond-stabilized polymer Muc2 network of C-terminal dimers and N-terminal trimers (15). Partial proteolysis by host proteases, which is visible in our denaturating gels of fecal mucin, does not change the structure of this network (15). However, splitting of S-S bonds dissolves it (i.e. mucolysis) resulting in reduced viscosity and increased permeability of the mucus layer (15). Typical S-S breaking agents are N-acetyl-cysteine (NAC), used in the treatment of cystic fibrosis, L-cysteine and 2-mercaptoethanol, all known to decrease mucin viscosity, *in vivo* and *in vitro* (16). Our DTNB results show that sulfide, compared with these thiols, is two-fold more potent in breaking S-S bonds. The pK_a of hydrogen sulfide is about 1 unit lower than that of the thiols, implying that the concentration of the nucleophilic agent (i.e. the anion) in the splitting of S-S is higher with hydrogen sulfide. Moreover, as sulfide donates 2 electrons it splits 2 S-S bonds, whereas thiols only split one. Elaborating on Ellman's mechanism of S-S splitting (17), we reasoned that the highly nucleophlic persulfide (30), formed in de first reaction, generates a trisulfide bond in the second one. Our *in vitro* results show that trisulfide formation is indeed specific for S-S reduction by sulfide. Our fecal analysis shows that trisulfide is also formed *in vivo* and stimulated by dietary heme, probably because bacterial sulfate reduction is heme dependent (19). Abx strongly reduce overall bacterial abundance and suppress this trisulfide formation almost completely, supporting our mechanism that trisulfide is formed by bacterial sulfide. In line with this, Abx greatly increased fecal excretion of Muc2 in a high MW polymeric form, as shown by nonreducing SDS-PAGE. Moreover, this polymeric Muc2 dissociates almost completely after reduction by DTT or sulfide, supporting the hypothesis that S-S bond splitting by sulfide opens the mucus barrier. This is supported further by the recent finding that increasing the number of S-S bonds in the Muc2 network increases the mucus barrier in mouse colon (24).

Our mechanism of S-S bond splitting by sulfide is supported by a recent nutritional study by Devkota *et al.* (25), showing that mono-association of germ-free mice with the sulfide-producing proteobacterium *Bilophila wardsworthia*, in the presence of taurocholate, results in breaking of the mucus barrier. The authors suggest that this is either due to sulfide (produced from taurine) or to unconjugated deoxycholate. However, the authors did not find barrier breaking in the presence of glycocholate, which is also metabolized to deoxycholate but does not generate sulfide. Therefore, we feel that their results can only be explained by the action of taurine-derived sulfide opening the mucus barrier via a mechanism analogous to what we propose here. Thus, it would be worthwhile to measure fecal trisulfides in that study.

Also in humans the colonic mucus layer functions as a barrier. As in mice, it prevents bacterial colonization of the epithelial surface and protects the surface cells from exposure to luminal toxic compounds (26). Three prevalent microbial profiles, so-called 'enterotypes' have been proposed to exist in human microbiota (27). Interestingly, for two of those enterotypes mucin-degrading bacteria are identified as microbial drivers. One enterotype is rich in *Prevotella* and the co-occurring *Desulfovibrio*. *Prevotella* degrades mucin and *Desulfovibrio* may enhance the rate limiting sulfatase step by hydrolyzing glycosyl-sulfate esters. The second mucin-degrading enterotype is rich in *Ruminococcus* and *Akkermansia*, both able to degrade mucins. We showed previously that dietary heme drastically increases the abundance of *Prevotella* and *Akkermansia* (10), which may be of relevance for these two enterotypes. The third enterotype is rich in *Bacteroides* using carbohydrates and proteins as substrates for fermentation (27). It would be of interest to see whether mucus-barrier differences between different enterotypes exist or that diseases of the gut, such as colorectal cancer and IBD, are associated with mucin-degrading enterotypes. Notably, in some people the colon microbiota do not reduce sulfate to hydrogen sulfide (28). Nevertheless, also in these people our mechanism of mucus barrier breaking may be of relevance because sulfide can be produced by bacteria from cysteine (e.g. *Prevotella*) or from taurine-conjugated bile acids, see (29) for review. In humans, and in our mice on a Western diet, the glycine/taurine ratio of bile acid conjugation is about 3 (30), implying that about 25% of the bile acids spilled over into the colon contain taurine.

Overall, we conclude that the microbiota facilitates the heme-induced hyperproliferation by opening the mucus barrier. Bacterial hydrogen sulfide can reduce the S-S bonds in polymeric mucin, thereby increasing the mucus layer permeability for mucin-degrading bacteria and for cytotoxic micelles. Consequently, epithelial surface cells are injured by the cytotoxic

heme and compensatory hyperproliferation is initiated. This hyperproliferation might eventually lead to colorectal cancer (20). Our model, as well as our results imply that fecal trisulfides can serve as a suitable marker of colonic mucolysis. Therefore, it would be of interest to measure levels of trisulfide in the human enterotypes, mentioned above, and in gut diseases in which the mucus barrier is compromised, such as IBD (31).

Materials and methods

Animal handling and design of the study. Experiments were approved by the Ethical Committee on Animal Testing of Wageningen University and were in accordance with national law. Eight week-old male C57BL6/J mice (Harlan, Horst, The Netherlands) were housed individually in a room with controlled temperature (20-24°C), relative humidity (55%±15%) and a 12h light dark cycle. Mice were fed diets and demineralized water ad libitum. Mice (n=9/group) received either a 'Westernized' control diet (40 en% fat (mainly palm-oil), low calcium (30 µmol/g)) or this diet supplemented with 0.5 µmol/g heme for 14 days, as previously described (32). Broad-spectrum antibiotics (Abx), containing ampicillin (1 g/L), neomycin (1 g/L), and metronidazole (0.5 g/L), were administered in drinking water during the time of intervention. There were 4 experimental groups; control, heme, control plus Abx and heme plus Abx. Feces were quantitatively collected during days 11-14, frozen at -20°C and subsequently freeze-dried. After 14 days, the colon was excised, mesenteric fat was removed and the colon was opened longitudinally, washed in PBS, and cut into three parts. The middle 1.5 cm colon tissue was formalin-fixed and paraffin embedded for histology. The remaining proximal and distal parts were scraped, pooled per mouse, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Colonic contents were sampled for microbiota analysis. Chemicals were from Sigma-Aldrich, unless indicated otherwise.

Fecal analyses. Fecal water was prepared by reconstituting freeze-dried feces with double distilled water to obtain a physiological osmolarity of 300 mOsm/l, as described previously (5). Cytotoxicity of fecal water was quantified by potassium release from human erythrocytes after incubation, as previously described (5) and validated with human colon carcinoma-derived Caco-2 cells (33). See Supplementary Methods for TBARS, bile acids and fecal mucin measurements.

Immunohistochemistry. Histological H&E and immunohistochemical Ki67 (6) and Slpi (34) stainings were performed on paraffin embedded colon sections as described previously. To quantify Ki67-positive colonocytes, 15 crypts per animal were counted. The number of Ki67-positive cells per crypt, total number of cells per crypt and labeling index were determined.

RNA isolation and microarray analysis. RNA was isolated from colon scrapings and hybridized on Affymetrix GeneChip Mouse Gene 1.1 ST arrays (for details see Supplementary Methods). Genes satisfying the criterion of false discovery rate <1% (q-value<0.01) were considered significantly expressed. Array data were submitted to the Gene Expression Omnibus, accession number GSE40670.

Bacterial DNA extraction and qPCR. DNA was extracted from approximately 0.1 g of fresh fecal pellet from the colon using the method described by Salonen et al (35). By qPCR total bacteria were quantified using generic 16S rRNA primers and Bacteroidetes and Firmicutes using phylum-specific 16S rRNA primers. See supplementary Methods for details.

Reduction of disulfide (S-S) bonds. S-S splitting potency of sodium sulfide (Na_2S) and thiols was determined using DTNB as model disulfide compound. Western blot analysis of fecal mucin was performed to determine whether DTT or sulfide reduces disulfide bonds in Muc2. See supplementary Methods for details.

Measurement of trisulfide bonds. By GC-MS total bound sulfides and acid-labile sulfides were measured in each individual fecal water. Trisulfides were calculated as the difference between those two. See supplementary Methods for details.

Statistics. *In vivo* data are presented as mean ± SEM. Differences between groups were tested for main effects by two-way ANOVA. *In vitro* data are given as mean ± SD and differences between groups were tested by one-way ANOVA with Bonferroni's Multiple Comparison Test. P-values <0.05 were considered significant.

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