

Hemoglobin and hemin induce DNA damage in human colon tumor cells HT29 clone 19A and in primary human colonocytes

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Abstract

Epidemiological findings have indicated that red meat increases the likelihood of colorectal cancer. Aim of this study was to investigate whether hemoglobin, or its prosthetic group heme, in red meat, is a genotoxic risk factor for cancer. Human colon tumor cells (HT29 clone 19A) and primary colonocytes were incubated with hemoglobin/hemin and DNA damage was investigated using the comet assay. Cell number, membrane damage, and metabolic activity were measured as parameters of cytotoxicity in both cell types. Effects on cell growth were determined using HT29 clone 19A cells. HT29 clone 19A cells were also used to explore possible pro-oxidative effects of hydrogen peroxide (H₂O₂) and antigenotoxic effects of the radical scavenger dimethyl sulfoxide (DMSO). Additionally we determined in HT29 clone 19A cells intracellular iron levels after incubation with hemoglobin/hemin. We found that hemoglobin increased DNA damage in primary cells ($\geq 10 \mu\text{M}$) and in HT29 clone 19A cells ($\geq 250 \mu\text{M}$). Hemin was genotoxic in both cell types (500–1000 μM) with concomitant cytotoxicity, detected as membrane damage. In both cell types, hemoglobin and hemin ($\geq 100 \mu\text{M}$) impaired metabolic activity. The growth of HT29 clone 19A cells was reduced by 50 μM hemoglobin and 10 μM hemin, indicating cytotoxicity at genotoxic concentrations. Hemoglobin or hemin did not enhance the genotoxic activity of H₂O₂ in HT29 clone 19A cells. On the contrary, DMSO reduced the genotoxicity of hemoglobin, which indicated that free radicals were scavenged by DMSO. Intracellular iron increased in hemoglobin/hemin treated HT29 clone 19A cells, reflecting a 40–50% iron uptake for each compound. In conclusion, our studies show that hemoglobin is genotoxic in human colon cells, and that this is associated with free radical mechanisms and with cytotoxicity, especially for hemin. Thus, hemoglobin/hemin, whether available from red meat or from bowel bleeding, may pose genotoxic and cytotoxic risks to human colon cells, both of which contribute to initiation and progression of colorectal carcinogenesis.

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1. Introduction

Experimental data in humans have shown that high consumption of red and processed meat and alcohol, in

combination with low consumption of vegetables and lack of exercise, coupled with genetic predisposition, increase the risk of developing colorectal cancer [1–4]. Pierre et al. have reported that hemoglobin and hemin given in a low-calcium diet to rats promote colorectal carcinogenesis at the aberrant crypt stage [5]. Recent results from human studies also suggest that intake of dietary heme iron is associated with an increased risk of prox-

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imal colon cancer, especially among women who drink [6]. Related to this is at least one finding that carriers of gene mutations (C282Y or H63D) for hemochromatosis (HFE), an autosomal recessive disease associated with increased body iron stores, have a significantly increased risk for developing colon cancer [7]. In extension of this, however, another study could not find the same association for the most common germ line mutations in the HFE gene, namely C282Y or H63D, whereas, there was a trend for an association in carriers of C282Y and H63D compound heterozygosity (C282Y/wild type and H63D/wild type) [8].

Meat, alcohol and ferrous iron are suspected of increasing the formation of free radicals in the bowel, especially of reactive oxygen species [9–11]. These radicals may damage the cells of the colon crypt or enhance tumor progression [12]. We have previously shown that human colon cell lines, treated with 250 μM ferric iron nitrilotriacetate (Fe-NTA) for 15 min to 24 h rapidly absorbed iron. Moreover, Fe-NTA (250–1000 μM) induced DNA breaks and oxidized DNA bases, which were enhanced by subsequent H_2O_2 exposure [13]. We also demonstrated that hemoglobin was as effective as Fe-NTA in inducing DNA damage [13]. We had, however, not investigated whether iron from hemoglobin or from its iron-containing prosthetic group, heme, was absorbed by the colon cells, and whether the induced DNA damage was associated with the generation of free radicals. These types of qualitative and quantitative data on the genotoxic impact of iron in the gut are needed to determine risk potentials. In the present study, we therefore investigated whether hemoglobin and hemin (oxidized heme) induce DNA damage in human colon cells and whether the resulting genotoxicity is related to generation of oxidative stress and to free radical formation. In addition to using HT29 clone cells, as in our first study, here we also used primary human colon cells. These non-transformed primary cells are relevant targets for the study of colon cancer risk compounds. Such cells are especially suited to assess genotoxicity related to initiation of carcinogenesis [14].

Using HT29 clone 19A cells, we also studied some possible mechanisms of activities, such as pro-oxidative activities mediated by the physiologically abundant peroxide H_2O_2 , or radical-scavenging activities mediated by the model compound DMSO, and the intracellular uptake of iron from both compounds using our in vitro culture conditions. Altogether the studies were expected to give more information on the genotoxic potential of hemoglobin, or its prosthetic group heme. Under given exposure situations (e.g. bleeding, high intake of red

meat) this activity of hemoglobin is expected to contribute to initiation and progression of colorectal carcinogenesis.

2. Materials and methods

2.1. Human colon cells and in vitro conditions

HT29 clone 19A is a permanently differentiated sub-clone derived from the carcinoma cell line HT29 after sodium butyrate treatment [15]. HT29 clone 19A cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Under given laboratory conditions HT29 clone 19A cells doubled their number within 24 h. Passages 27–48 were used for the experiments.

Primary colon cells were freshly isolated from colon tissue obtained during surgical resections, as described previously [16]. The donors (n : nine males, age: 69 ± 9.4) of this colon tissue had given their informed consent and were admitted to the hospital for colorectal surgery. Non-tumorous tissue for cell isolation was excised together with tumor tissue for medical indications. The study was approved by the Ethical Committee of the Friedrich-Schiller-University Jena.

Bovine hemoglobin (which predominantly consists of methemoglobin since native hemoglobin is rapidly oxidized; Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in cell culture medium RPMI 1640 (Life Technologies, Karlsruhe, Germany), DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin, or minimum essential medium (MEM). The solutions in RPMI/DMEM or MEM were used for experiments with HT29 clone 19A and primary cells, respectively. Hemin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was prepared in a stock solution in 20 mM sodium hydroxide (NaOH) (Fisher Scientific, Loughborough, UK) and further diluted in the appropriate cell culture medium. Colon cells were incubated with hemin or hemoglobin at 37 °C at different concentrations (10–1000 μM) or for different periods of time as is specified in the legends of the figures and tables.

2.2. Detection of DNA damage

DNA damage was measured using single cell microgel-electrophoresis, as has been described in detail by Glei et al. [13]. Microscopical analysis revealed images of damaged DNA (“comets”). The proportion and extent of DNA migration were determined for 50 images per slide using the image analyzing system of Perceptive Instruments (Suffolk, UK, www.perceptive.co.uk). The intensity of fluorescence in the comet tail, expressed as % fluorescence in tail, was used as the evaluation criteria. For each data point mean values of three parallel slides of one experiment were the basis for calculating overall mean values of independently reproduced experiments ($n = 3–5$), as is specified in the tables and figures.

2.3. Cytotoxicity determined as metabolic activity and cell growth

The influence of hemoglobin and hemin on metabolic activity/viability and cell growth was determined in 96-well microtiter plates. Colon cells were treated with hemoglobin (0.1–1000 μM) or hemin (1–1000 μM) in culture medium for 24 and 72 h (only HT29 clone 10A cells were used for experiments lasting for 72 h). After adding 20 μl CellTiter-Blue™ (Promega, Mannheim, Germany) reagent 2 h before the end of the incubation period, resazurin was converted into resorufin by metabolic active cells. The intensity of the resulting fluorescence was measured with Ex/Em 520/595 nm. In further experiments, DNA content was assessed in HT29 clone 19A cells by fixing and permeabilizing the cells with methanol for 5 min, and then adding 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) which binds to DNA. After 30 min, the DNA content per well was detected, as a reflection of the remaining cells, using fluorimetric analysis with Ex/Em 360/450 nm. Mean values (three parallel determinations per experiment, 3–6 experiments) were recorded for final evaluation.

2.4. Analysis of iron content

HT29 clone 19A cells were incubated with 250 μM hemoglobin or 32 μM hemin for a period beginning from 15 min to 24 h. The iron content of the culture medium and cells were analyzed separately. This was done using inductively coupled argon plasma emission spectrometry (Liberty Serie II ICP-AES, Varian, Darmstadt) as described previously [13].

2.5. Statistical evaluation

Data shown in the tables and figures represent mean values \pm S.D. Unless otherwise stated, these mean values of at least three independent experiments were calculated from the means of triple replicates obtained in each experiment. Statistical evaluation was performed with GraphPad Prism Version 3.0 and 4.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Depending on sample size and type of experiment, *t*-test or one-way ANOVA was used to determine significance of the experimental variables. The significance of individual treatment groups in comparison to the controls was determined with the Bonferroni's multiple comparison post test (with selected pairs). EC_{50} was calculated with non-linear regression and one phase exponential decay. The statistical analyses used were dependent on the respective experimental design and are specified in the legends of the figures and tables.

3. Results

3.1. Genotoxicity

Both compounds, hemoglobin and hemin, significantly induced DNA damage in HT29 clone 19A cells with no apparent differences in the genotoxic potency of the two compounds at concentrations up to 500 μM (Table 1). At the higher concentration, hemin was cytotoxic and decreased the cell viability more in HT29 clone 19A cells than in primary colon cells. Hemin can therefore not be defined as being genotoxic at this

Table 1

Hemoglobin and hemin (15 min incubation, 37 °C) induced DNA damage (tail intensity %) in primary colon cells (unpaired *t*-test, §*p* < 0.05) and in HT29 clone 19A cells (*n* = 4–5; one-way ANOVA, ***p* < 0.01, ****p* < 0.001, Bonferroni's multiple comparison test to control, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *n* = 3). Simultaneously hemin decreased the cell viability in HT29 clone 19A (Bonferroni's multiple comparison test to control, ****p* < 0.001, *n* = 3)

Primary cells				HT29									
Fe-source [μM]	Viability [%]			Tail intensity [%]			Fe-source [μM]	Viability [%]			Tail intensity [%]		
	Mean	S.D.	ns	Mean	S.D.	ns		Mean	S.D.	ns	Mean	S.D.	**
Hemoglobin	0	89.6	3.5	3.7	2.2		0	93	5		3.1	0.4	
	10	93.9	2.4	11.7§	5.7		10	92	5		4	0.9	
	100	86.8	8.4	13.8	7.8		100	93	4		5.5	1.6	
	250	90.0	3.9	12.9§	5.9		250	94	2		7.4	1	
	500	86.0	5.2	12.9§	4.9		500	92	5		9.3	0.5	*
1000	90.0	2.4	13.2§	4.9		1000	92	5		9.9	4.5	**	
Hemin	Mean	S.D.	ns	Mean	S.D.	**	Hemin	Mean	S.D.	***	Mean	S.D.	***
	0	90.8	2.9	3.6	1.2		0	91	5		3.4	0.8	
	10	90.8	4.3	7.9	1.3		10	91	4		4	0.8	
	100	92.7	2.6	7.7	2.9		100	89	8		3.5	0.3	
	250	91.4	6.3	10.5	6.2		250	90	6		4.7	1.1	
	500	88.8	5.4	8.4	1.8		500	70	17		10.1	5.9	
1000	86.8	8.8	12.0	4.8	*	1000	40	20	***	22.9	8.2	***	

Table 2
Effects of hemoglobin and hemin on metabolic activity in primary cells and HT29 clone 19A cells after 24 h treatment

Hemin (μM)	Primary cells				HT29				Hemoglobin (μM)	Primary cells				HT29			
	Mean	S.D.	n	***	Mean	S.D.	n	***		Mean	S.D.	n	***	Mean	S.D.	n	***
0	100	0	4		100	0	3		0	100	0	2		100	0	3	
100	90	8	4	**	97	8	3		100	70	4	3	***	69	4	3	***
250	75	9	4	***	81	0	3	***	250	54	7	3	***	48	2	3	***
500	69	4	4	***	66	5	3	***	500	43	9	3	***	36	2	3	***
1000	51	12	4	***	47	5	3	***	1000	22	6	3	***	22	2	3	***

The activity of the medium control was set to equal 100%. Hemoglobin and hemin significantly decreased the metabolic activity (one-way ANOVA, *** $p < 0.001$). Significant differences to the medium control are indicated with asterisks (** $p < 0.01$, *** $p < 0.001$). No differences were detectable between primary cells and the carcinoma cell line.

concentration ($>500 \mu\text{M}$), since false positive results (increased DNA damage) due to DNA fragmentation in dying cells cannot be excluded [17,18].

Initial DNA-damage is not different between HT29 clone 19A cells and primary colon cells. In the latter cells, hemin significantly induced DNA damage at $1000 \mu\text{M}$ whereas hemoglobin already caused DNA-damage at low concentrations ($10 \mu\text{M}$), however, without revealing a concentration-response relationship.

3.2. Metabolic activity

Metabolic activity was measured in HT29 clone 19A and in primary colon cells after 24 h incubation with hemoglobin and hemin (Table 2). Both compounds ($\geq 100 \mu\text{M}$) significantly reduced the metabolic activity with increasing concentrations, indicating cytotoxic effects. Both cell types were of similar sensitivity, without any significant differences between them.

3.3. Cell growth

Table 3 shows that both hemoglobin and hemin reduced the cell number of HT29 clone 19A cells after 24 and 72 h treatment. Hemin was markedly more effective than hemoglobin. The calculated EC_{50} values for 24 h of incubation were $91 \mu\text{M}$ (hemin) and not detectable for hemoglobin. After 72 h, the corresponding values were $76 \mu\text{M}$ (hemin) and $921 \mu\text{M}$ (hemoglobin). There were no significant differences between the two different durations of exposure (24 and 72 h), thus the induction of this particular cytotoxic effect did not seem to depend on time.

3.4. Iron- H_2O_2 interaction

Next, we investigated the genotoxic potentials of hemoglobin and hemin in combination with H_2O_2 to determine possible pro-oxidative activities. For this, we pre-treated (15 min, 37°C) the HT29 clone 19A

Table 3
Effects of hemoglobin and hemin on HT29 clone 19A cell number after 24 and 72 h

Hemin (μM)	24 h				72 h				Hemoglobin (μM)	24 h				72 h			
	Mean	S.D.	n	***	Mean	S.D.	n	***		Mean	S.D.	n	***	Mean	S.D.	n	***
0	100	0	3		100	0	6		0	100	0	3		100	0	4	
1	96	4	3		111	12	6		1	97	2	3		98	13	4	
10	80	0	3	***	89	9	6		10	92	5	3		88	13	4	
50	61	2	3	***	57	7	6	***	50	90	2	3	*	81	16	4	
100	49	1	3	***	47	8	6	***	100	86	4	3	***	80	20	4	
250	33	2	3	***	28	5	6	***	250	74	4	3	***	73	17	4	*
500	25	1	3	***	20	5	6	***	500	66	4	3	***	62	11	4	**
750	19	5	3	***	18	4	6	***	750	65	4	3	***	55	9	4	***
1000	16	3	3	***	22	7	6	***	1000	54	4	3	***	46	5	4	***

The cell number of the medium control was set to equal 100%, thus each value represents the relative cell number. Hemoglobin and hemin decreased the cell number significantly (one-way ANOVA, *** $p < 0.001$). Significant differences to the medium control are indicated with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 4

Combination effects of hemoglobin or hemin (15 min pretreatment of cells at 37 °C) and H₂O₂ (added to cells on slides, 5 min, 4 °C) on DNA damage (tail intensity %) in HT 29 clone 19A cells

Fe-source (μM)	H ₂ O ₂ (μM)					H ₂ O ₂ (μM)					H ₂ O ₂ (μM)						
	0		18.75		***, ###	0		9.37		***, ###	0		4.69		###		
	Mean	S.D.	Mean	S.D.		Mean	S.D.	Mean	S.D.		Mean	S.D.	Mean	S.D.			
Hemoglobin																	
0	2.9	0.9	10.5	2.0	***	2.5	0.2	6.9	2.2	*	3.0	0.2	6.5	0.9			
10	3.9	0.4	9.3	2.2	*	4.3	1.1	6.3	1.6		4.2	1.6	6.7	1.5			
100	7.5	3.2	#	9.4	1.3		6.2	1.5	#	7.5	1.3		6.2	2.2	8.7	0.7	
250	9.6	0.5	###	11.7	3.3		9.3	1.6	###	11.0	1.5	##	10.9	2.4	##	12.7	2.9
500	13.4	2.3	###	15.8	3.0	##	14.2	2.2	###	16.9	2.1	###	13.5	4.2	###	14.2	2.8
1000	13.2	1.0	###	18.4	0.1	*, ###	14.8	1.1	###	18.5	1.3	###	16.4	4.9	###	16.1	3.7
Hemin																	
0	4.1	1.0	9.1	1.8		2.4	0.4	4.6	1.4		2.5	0.2	5.3	1.6			
10	4.2	1.1	7.6	0.8		2.9	0.7	4.2	0.6		2.9	0.6	7.1	3.6			
50	5.5	0.2	9.3	2.0		3.9	0.6	7.5	1.2		4.5	2.3	8.5	3.3			
100	5.5	0.5	10.9	0.8		4.1	1.6	9.4	4.6	*, #	5.2	2.4	11.3	6.8			
250	11.1	5.1	#	16.7	4.6	#	7.2	1.5	#	8.9	1.5	#	5.9	0.9	9.8	5.8	
500	20.9	7.2	###	26.4	3.1	###	12.5	1.4	###	17.8	3.8	*, ###	9.3	3.4	11.5	5.0	

Means and S.D. from $n=3$ independently reproduced experiments are presented. Significant differences to control without iron is indicated by #, significant differences to corresponding incubation with hemoglobin/hemin and without H₂O₂ are indicated by * (Two-way ANOVA, ***,### $p < 0.001$, ** $p < 0.01$; Bonferroni's multiple comparison test against control, * $p < 0.05$, ** $p < 0.01$, ***,### $p < 0.001$).

cells in suspension with 10–1000 μM hemoglobin and 10–500 μM hemin, respectively. Afterwards, the cells (in agarose gel on slides) were damaged with low genotoxic doses of H₂O₂ (4.69–18.75 μM, 5 min, 4 °C). The combination treatment resulted in significant increases of DNA damage at only few data points (Table 4). Altogether the results merely reflected marginal additive effects by H₂O₂. Significant differences for the various treatment options were not detected.

3.5. Effects of the radical scavenger DMSO on DNA damage

DMSO is a well-known scavenger of free radicals, and thus may be used to indirectly elucidate radical mechanisms of toxicity [19]. Fig. 1 clearly demonstrates that DNA damage caused by hemoglobin and by H₂O₂ (used as a positive control on account of its radical generating properties) is clearly reduced in the presence of increasing DMSO concentrations. These results support the hypothesis that radicals formed by hemoglobin and H₂O₂ are scavenged by DMSO.

3.6. Cellular iron uptake

Iron from both hemoglobin and hemin was rapidly taken up by HT29 clone 19A cells. A significant time-effect relationship was observed. This response curve

did not reach its plateau at the end of the experiment, namely after 24 h, for either of the two compounds. At the same time, total iron concentrations in the medium were reduced. This resulted in the finding that the sum of the curves obtained for cellular and medium-concentrations revealed steady state kinetics for both Fe-donors (data not shown). The absolute iron concentrations (μg per ml or per million cells) were approximately 10-fold different for the two compounds, since 10-fold different concentrations (32 μM for hemin and 250 μM for

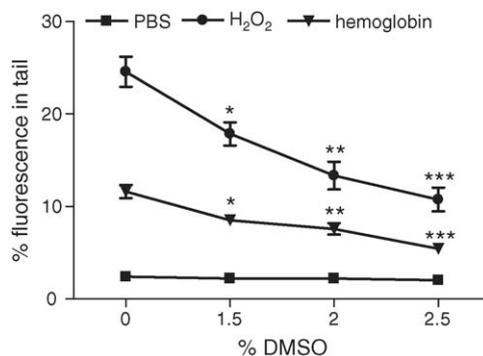


Fig. 1. Significant reduction (one-way ANOVA $p < 0.001$, Bonferroni's multiple comparison test against control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) of DNA damage after adding increasing concentrations of the radical scavenger DMSO to cell suspensions treated with genotoxic concentrations of hemoglobin (1000 μM, 15 min, 37 °C) or H₂O₂ (37.5 μM, 5 min, 4 °C).

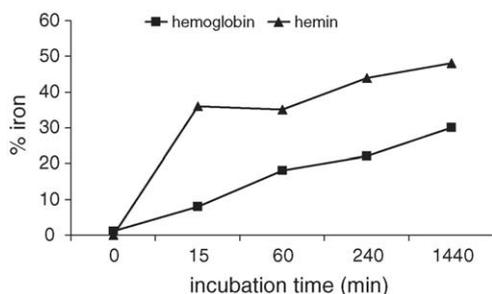


Fig. 2. Rates of cellular iron uptake by HT29 clone 19A cells incubated with 32 μM hemin or 250 μM hemoglobin for 15 min to 24 h. There was a clear time dependent increase of the cellular iron content with a higher relative level after hemin treatment.

hemoglobin) were originally added to the incubation mixtures to account for their different cytotoxic potentials. The comparison of iron uptake by the cells incubated with approximately equitoxic concentrations of both iron sources revealed that more Fe was available from hemin than from hemoglobin (Fig. 2).

4. Discussion

High intake of red meat is most likely associated with increased colon cancer risk, as has first been reported in prospective studies by Willett et al. [20], and then in later reviews of observational and experimental studies and of two meta-analyses [21]. Results of other studies indicated that high consumption of red meat, or of processed meat, in particular, might be associated with an increased risk of cancer of the large intestine [22,23]. Most recently, members of “The European Prospective Investigation into Cancer and Nutrition” (EPIC) study group have provided new information on the intake of meat and colorectal cancer risks [24]. 478,040 men and women from 10 European countries were prospectively followed up who were free of cancer at the time of enrollment between 1992 and 1998. After a mean follow-up of 4.8 years, 1329 incident colorectal cancers were documented and, among others, the relationship was examined between intakes of red and processed meat and colorectal cancer risk. The analysis confirmed the previous studies that colorectal cancer risk is positively associated with high consumption of red and processed meat. The authors reported that the overall association with colorectal cancer risk was, however, stronger for processed meat, than for unprocessed red meat. They discussed this on the basis of heme, which was present in all of the red meat and virtually all of the processed meat studied. Since heme was shown to stimulate production of endogenous *N*-nitroso compounds in the human gas-

trointestinal system [25] the endogenous *N*-nitrosation, arising from ingestion of heme, was suggested to account for the increased risk of colorectal cancer of the EPIC study.

In the present paper, we have instead hypothesized that hemoglobin and possibly hemin may have other modes of activities in the gut lumen subsequent to red and processed meat intake. Hemoglobin or myoglobin probably release the globin to yield an intact heme complex which then may be absorbed by the intestinal mucosa [26]. Additionally, significant quantities of the dietary heme may be degraded to yield inorganic iron complexes and may be subsequently absorbed in that form [26]. Under *in vivo* conditions, unabsorbed dietary iron in the gut lumen may be available for Haber–Weiss and Fenton-type reactions to yield radicals from peroxides, which in turn may be genotoxic and pose risks of colorectal cancer [9,10,27]. In accordance with this hypothesis, we have now demonstrated in the present study that hemoglobin is genotoxic.

The genotoxicity of hemoglobin was observed at sub-toxic concentrations, whereas, DNA damage, induced by hemin, was detected only with concomitant cytotoxicity. In HT29 clone 19A cells, as well as in primary colon cells, hemoglobin (up to 1000 μM , 15 min of exposure) was non-cytotoxic according to the trypan blue exclusion assay, which measures membrane damage. In contrast, hemin was cytotoxic in the trypan blue exclusion assay at 1000 μM , but only in HT29 clone 19A cells and not in primary colon cells. These findings support conclusions from studies with rats fed purified diets supplemented with hemin, which showed that fecal water from these rats had cytolytic properties [28,29]. The different cytotoxic potential between hemoglobin and hemin was also apparent in another test system, which detected cytotoxicity by measuring the impairment of cell growth (and which could only be performed in HT29 clone 19A cells, since primary cells do not proliferate in culture). Here, only 10 μM hemin, but 50–100 μM hemoglobin, were needed to significantly impair cell growth. The differences between the cytotoxic potentials, and especially the cause of the relatively lower membrane damaging potential of hemoglobin in HT29 clone 19A cells, could be due to the kinetics of a gradual release of toxic hemin from hemoglobin. The lack of membrane damaging cytotoxicity in primary cells may be due to a better cellular uptake of hemin by mechanisms that are more expressed in primary colon cells [26,30], than in tumor cells. When assessing cytotoxicity in both cell types by measuring metabolic activity, the differences between the cytotoxic potentials of the compounds are not as apparent. In this case, already low concentrations

of both compounds ($\geq 100 \mu\text{M}$) reduced metabolic activity in HT29 clone 19A cells and in primary colon cells. This may reflect different cellular kinetics for intracellular toxic activities in comparison to extracellular caused membrane damage.

Genotoxic effects were caused by hemin in both cell types at $1000 \mu\text{M}$, which was a cytotoxic concentration according to the measurements used in this study. In contrast, hemoglobin was genotoxic also at lower concentrations ($\geq 10 \mu\text{M}$ in primary colon cells, $\geq 500 \mu\text{M}$ in HT29 clone 19A cells). These concentrations were non-cytotoxic in primary human colon cells, which here were used as models for possible mechanisms related to initiation of cell transformation. It is important to relate the employed concentration ranges and the genotoxic/cytotoxic effective concentrations of hemoglobin and hemin to the possible exposures found in the gut lumen. One thousand micromolars was the highest concentration that has been tested. This amount is only three-fold more than the levels that had been reported to occur in the gut lumen after iron supplementation. Thus, the genotoxic dose of only $10 \mu\text{M}$ is well within the physiological concentration range [12,31]. For example, Lund et al. reported that the concentration of water-soluble iron in the gut lumen is normally around $25 \mu\text{M}$, but can rise to $>100 \mu\text{M}$ in human feces, with a total concentration of iron in the intraluminal pool reaching $350 \mu\text{M}$ after oral supplementation of ferrous sulfate [12,31]. Pierre et al. fed rats (treated with azoxymethane) with meat containing low, medium and high heme levels and with ferric citrate and hemoglobin [32]. They found that the heme content in freeze-dried feces and fecal water correlated to the intake and reached levels of $19\text{--}1097 \mu\text{M}$. In a study by Sesink et al., the feeding of rats with a purified diet supplemented with $1.3 \mu\text{mol/g}$ of hemin resulted in significantly higher fecal levels of iron ($257 \mu\text{M}$) than in the controls ($80 \mu\text{M}$) [28]. Thus in both cases the reported fecal iron concentrations were also within our cytotoxic and genotoxic concentration ranges.

Our studies additionally provide evidence, that hemoglobin also exerts genotoxic effects via the generation of free radicals, since its genotoxicity was markedly impaired in the presence of increasing concentrations of the radical scavenger DMSO. It is possible that reactive oxygen species were formed from peroxides or lipids by the catalytic activity of heme iron [33,34]. Also, radicals arising directly from hemoglobin have been reported to occur [35]. With our studies, it cannot be estimated at which proportion hydroxyl radicals were generated. They should cause pro-oxidative activities with H_2O_2 , which was not observed here. The finding however points to a possible mechanism of dietary chemoprotection

against iron-mediated effects in the colon, in vivo. On the basis of generally accepted knowledge, it could now be expected that consumption of vegetable and fruits, which are high in antioxidant food ingredients, could counteract the activities of iron in the gut by scavenging free radicals. This has not directly been demonstrated yet. Indirectly, however, it has been shown that the fecal matrix is capable of generating reactive oxygen species in abundance [11]. The free radicals may be dietary-related since another group demonstrated an enhanced formation of free radicals in fecal water of subjects consuming a diet rich in red meat and fat. There was a 13-fold higher production of reactive oxygen species in comparison to fecal water from the same individuals who in a later intervention phase consumed a diet high in dietary plant foods [36]. Using the comet assay, we studied the same fecal waters for their genotoxic activities in human colon cells. We were able to observe a reduced level of DNA damaging agents in the fecal water from the vegetable intervention phase in comparison to the fecal waters derived during the high meat/fat consumption period [37]. More recently we have performed an intervention trial with breads supplemented with prebiotics \pm antioxidants and determined different biomarkers of genotoxicity and oxidative DNA damage [38]. The measurements included fecal water genotoxicity, which reflects the exposure situation in the colon lumen. Fecal water genotoxicity was reduced in non-smokers although there was no detectable difference between fecal waters from subjects consuming prebiotic breads and those consuming breads additionally supplemented with antioxidants. Presently, these studies do not answer the specific question on whether dietary antioxidants are capable of reducing damage from hemoglobin-derived radicals in the human gut lumen. They do, however, point to the possibility that this mechanism could take place, although it will be necessary to perform more focused intervention trials in the future to resolve the issue.

According to the hypothesis, cellular absorption of iron is the basis for detecting genotoxic potentials of hemoglobin and hemin in human colon cells. The results presented here indeed do indicate that exposure with hemin and hemoglobin can lead to increased cellular concentrations of iron in HT29 clone 19A cells. After 15 min treatment, already 8 and 36% of the supplemented iron had been absorbed from hemoglobin and from hemin, respectively. Compared to this, the treatment of HT29 clone 19A cells with Fe-NTA ($250 \mu\text{M}$) resulted in an uptake of almost 50% of the same dose after 15 min [13]. These relative absorption values (Fe-NTA $>$ hemin $>$ hemoglobin) are inversely related to the

molecular weights of the compounds, which may be of importance for cellular bioavailability. Absorption of iron mainly takes place in the duodenum and proximal jejunum, a process, which is well controlled since specific iron-excretion pathways do not seem to exist. In rats fed purified control diets, or purified diets supplemented with 1.3 $\mu\text{mol/g}$ of hemin, protoporphyrin IX, ferric citrate, or bilirubin ($n = 8/\text{group}$) for 14 days Sesink et al. have studied iron absorption and effects of iron in the colon lumen. They showed that the apparent iron absorption (occurring in the small intestine) was about 12% of dietary intake in the control group and that the additional uptake of iron from supplemental hemin and ferric citrate was very low [28]. This means that the majority of dietary heme iron and iron from ferric citrate reached the colon. The authors were then able to conclude that heme iron and not inorganic iron was probably responsible for the fecal water cytotoxicity and increased colonic epithelial proliferation observed in that study.

Heme-iron is absorbed by the intestinal mucosa as the intact heme complex [26,30] and Fe (II) is then liberated in the cell via hemoxygenases [39]. Bioavailability of heme in humans was estimated to be up to 35% and at least 65% of total ingested heme iron reaches the colon (reviewed in [26]). In biological systems iron frequently exists as its insoluble ferric Fe (III) form, which may arise by decomposition of heme and which is thus also related to red meat. The cellular uptake of inorganic iron is mediated by transport systems, which require the presence of the ferrous Fe (II) ion, which is very unstable and quickly oxidizes to ferric iron. To enable absorption, specialized transmembrane electron transport systems evolved, known as ferric-chelate reductases. They function by reducing ferric Fe (III) to the ferrous Fe (II) form at the extracellular surface, thus allowing the cell to take up the ferrous iron [40]. This can then be transported into the cell by the transport protein DMT1 (divalent metal transporter 1) [41]. HT29 clone 19A cells express DMT1 [42], which contributes to the efficient cellular uptake we have demonstrated here. Whether or not primary colon cells, as used here, are better equipped to take up the heme complex from hemoglobin, as suggested by our findings, is not known and will need to be resolved in future studies.

In conclusion, the data here presented show that hemin was cytotoxic to human colon cells. There was also a marked genotoxic potential of hemoglobin in primary colon cells at non-cytotoxic concentrations. The studies of mechanisms revealed that hemin and hemoglobin increased iron concentrations in human colon tumor cells and that the observed genotoxicity is probably related to formation of radicals. Thus our stud-

ies give new experimental support for the hypothesis that red meat (as a source of hemoglobin, hemin, and iron) contributes to the carcinogenic process through initiation of non-transformed cells and enhanced progression of transformed cells.

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References

- [1] L.L. Marchand, J.H. Hankin, L.R. Wilkens, L.M. Pierce, A. Franke, L.N. Kolonel, A. Seifried, L.J. Custer, W. Chang, A. Lum-Jones, T. Donlon, Combined effects of well-done red meat, smoking, and rapid *N*-acetyltransferase 2 and CYP1A2 phenotypes in increasing colorectal cancer risk, *Cancer Epidemiol. Biomarkers Prev.* 10 (2002) 1259–1266.
- [2] M.-C. Boutron-Ruault, P. Senesse, S. Méance, C. Belghiti, J. Faivre, Energy intake, body mass index, physical activity and the colorectal adenoma-carcinoma sequence, *Nutr. Cancer* 39 (2001) 50–57.
- [3] E. Negri, C. La Vecchia, S. Franceschi, B. D'Avanzo, F. Parazzini, Vegetable and fruit consumption and cancer risk, *Int. J. Cancer* 48 (1991) 350–354.
- [4] M. Bardou, S. Montembault, V. Giraud, A. Balian, E. Borotta, C. Houdayer, F. Capron, J.-C. Chaput, S. Naveau, Excessive alcohol consumption favours high-risk polyp or colorectal cancer occurrence among patients with adenomas: a case control study, *GUT* 50 (2002) 38–42.
- [5] F. Pierre, S. Tache, C.R. Petit, R. Van der Meer, D.E. Corpet, Meat and cancer: haemoglobin and haemin in a low-calcium diet promote colorectal carcinogenesis at the aberrant crypt stage in rats, *Carcinogenesis* 24 (2003) 1683–1690.
- [6] D.H. Lee, K.E. Anderson, L.J. Harnack, A.R. Folsom, D.R. Jacobs Jr., Heme iron, zinc, alcohol consumption, and colon cancer: Iowa Women's Health Study, *JNCI Cancer Spectr.* 96 (2004) 403–407.
- [7] N.J. Shaheen, L.M. Silverman, T. Keku, L.B. Lawrence, E.M. Rohlfs, C.F. Martin, J. Galanko, R.S. Sandler, Association between hemochromatosis (HFE) gene mutation carrier status and the risk of colon cancer, *JNCI Cancer Spectr.* 95 (2003) 154–159.
- [8] J.P. Robinson, V.L. Johnson, P.A. Rogers, R.S. Houlston, E.R. Maher, D.T. Bishop, D.G. Evans, H.J. Thomas, I.P. Tomlinson, A.R. Silver, Evidence for an association between compound heterozygosity for germ line mutations in the hemochromatosis (HFE) gene and increased risk of colorectal cancer, *Cancer Epidemiol. Biomarkers Prev.* 14 (2005) 1460–1463.
- [9] J. Imlay, S.M. Chin, S. Linn, Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro, *Science* (1988) 640–642.

- [10] M. Tseng, R.S. Sandler, E.R. Greenberg, J.S. Mandel, R.W. Haile, J.A. Baron, Dietary iron and recurrence of colorectal adenomas, *Cancer Epidemiol. Biomarkers Prev.* 6 (1997) 1023–1029.
- [11] R.W. Owen, B. Spiegelhalter, H. Bartsch, Generation of reactive oxygen species by the faecal matrix, *GUT* 46 (2000) 225–232.
- [12] E.K. Lund, S.G. Wharf, S.J. Fairweather-Tait, I.T. Johnson, Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine, *J. Nutr.* 128 (1998) 175–179.
- [13] M. Gleis, A. Klinder, G.O. Latunde-Dada, T.W. Becker, U. Hermann, K. Voigt, B.L. Pool-Zobel, Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A, *Mutat. Res.* 519 (2002) 151–161.
- [14] B.L. Pool-Zobel, U. Leucht, Induction of DNA damage in human colon cells derived from biopsies by suggested risk factors of colon cancer, *Mutat. Res.* 375 (1997) 105–116.
- [15] C. Augeron, C.L. Laboisse, Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate, *Cancer Res.* 44 (1984) 3961–3969.
- [16] A. Schäferhenrich, W. Sendt, J. Scheele, A. Kuechler, T. Liehr, U. Claussen, A. Rapp, K.O. Greulich, B.L. Pool-Zobel, Endogenously formed cancer risk factors induce damage of *p53* in human colon cells obtained from surgical samples, *Fd Chem. Toxicol.* 41 (2003) 655–664.
- [17] B.L. Pool, S.Y. Brendler, U.M. Liegibel, A. Tompa, P. Schmezer, Employment of adult mammalian primary cells in toxicology: in vivo and in vitro genotoxic effects of environmentally significant *N*-nitrosodialkylamines in cells of the liver, lung and kidney, *Environ. Mol. Mutagen* 15 (1990) 24–35.
- [18] S.Y. Brendler-Schwaab, P. Schmezer, U.M. Liegibel, S. Weber, K. Michalek, A. Tompa, B.L. Pool-Zobel, Cells of different tissues for in vitro and in vivo studies in toxicology: compilation of isolation methods, *Toxicology* 8 (1994) 1285–1302.
- [19] T.K. Hei, S.X. Liu, C. Waldren, Mutagenicity of arsenic in mammalian cells: Role of reactive oxygen species, *Proc. Natl. Acad. Sci. U.S.A.* 95 (1998) 8103–8107.
- [20] W.C. Willett, M.J. Stampfer, G.A. Colditz, B.A. Rosner, F.E. Speizer, Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women, *N. Engl. J. Med.* 323 (1990) 1664–1672.
- [21] World Cancer Research Fund, American Institute for Cancer Research. *Food, Nutrition and the Prevention of Cancer: A Global Perspective*, American Institute for Cancer Research, Washington, DC, 1997.
- [22] E.K. Wei, E. Giovannucci, K. Wu, B. Rosner, C.S. Fuchs, W.C. Willett, G.A. Colditz, Comparison of risk factors for colon and rectal cancer, *Int. J. Cancer* 108 (2004) 433–442.
- [23] A. Chao, M.J. Thun, C.J. Connell, M.L. McCullough, E.J. Jacobs, W.D. Flanders, C. Rodriguez, R. Sinha, E.E. Calle, Meat consumption and risk of colorectal cancer, *JAMA* 293 (2005) 172–182.
- [24] T. Norat, S. Bingham, P. Ferrari, N. Slimani, M. Jenab, M. Mazuir, K. Overvad, A. Olsen, A. Tjønneland, F. Clavel, M.C. Boutron-Ruault, E. Kesse, H. Boeing, M.M. Bergmann, A. Nieters, J. Linseisen, A. Trichopoulos, D. Trichopoulos, Y. Tountas, F. Berrino, D. Palli, S. Panico, R. Tumino, P. Vineis, H.B. Bueno-de-Mesquita, P.H. Peeters, D. Engeset, E. Lund, G. Skeie, E. Ardanaz, C. Gonzalez, C. Navarro, J.R. Quiros, M.J. Sanchez, G. Berglund, I. Mattisson, G. Hallmans, R. Palmqvist, N.E. Day, K.T. Khaw, T.J. Key, J.M. San, B. Hemon, R. Saracci, R. Kaaks, E. Riboli, Meat, fish, and colorectal cancer risk: the European prospective investigation into cancer and nutrition, *J. Natl. Cancer Inst.* 97 (2005) 906–916.
- [25] A.J. Cross, J.R. Pollock, S.A. Bingham, Haem, not protein or inorganic iron, is responsible for endogenous intestinal *N*-nitrosation arising from red meat, *Cancer Res.* 63 (2003) 2358–2360.
- [26] C.E. Carpenter, A.W. Mahoney, Contributions of heme and non-heme iron to human nutrition, *Crit. Rev. Food Sci. Nutr.* 31 (1992) 333–367.
- [27] J. Everse, N. Hsia, The toxicities of native and modified hemoglobins, *Free Rad. Biol. Med.* 22 (1997) 1075–1099.
- [28] A.L.A. Sesink, D.S.M.L. Termont, J.H. Kleibeuker, R. Van der Meer, Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme, *Cancer Res.* 59 (1999) 5704–5709.
- [29] van Kraaij van der Meer, E. Kramer, D. Jonker-Termont, M.B. Katan, M.R. van der, J. Keijer, Differential gene expression in rat colon by dietary heme and calcium, *Carcinogenesis* 26 (2005) 73–79.
- [30] L.R. Weintraub, M.B. Weinstein, H.-J. Huser, S. Rafal, Absorption of hemoglobin iron: the role of a heme-splitting substance in the intestinal mucosa, *J. Clin. Invest.* 47 (1968) 531–539.
- [31] E.K. Lund, S.G. Wharf, S.J. Fairweather-Tait, I.T. Johnson, Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers, *Am. J. Clin. Nutr.* 69 (1999) 250–255.
- [32] F. Pierre, A. Freeman, S. Tache, M.R. van der, D.E. Corpet, Beef meat and blood sausage promote the formation of azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colons, *J. Nutr.* 134 (2004) 2711–2716.
- [33] J.L. Pierre, M. Fontecave, R.R. Crichton, Chemistry for an essential biological process: the reduction of ferric iron, *BioMetals* 15 (2002) 341–346.
- [34] B.J. Reeder, M.A. Sharpe, A.D. Kay, M. Kerr, K. Morre, M.T. Wilson, Toxicity of myoglobin and haemoglobin: oxidative stress in patients with rhabdomyolysis and subarachnoid haemorrhage, *Biochem. Soc. Trans.* 30 (2002) 745–748.
- [35] D. Ramirez, Y.R. Chen, R.P. Mason, Immunochemical detection of hemoglobin-derived radicals formed by reaction with hydrogen peroxide: involvement of a protein-tyrosyl radical, *Free Rad. Biol. Med.* 34 (2003) 830–839.
- [36] J.G. Erhardt, S.S. Lim, J.C. Bode, C. Bode, A diet rich in fat and poor in dietary fiber increases the in vitro formation of reactive oxygen species in human feces, *J. Nutr.* 127 (1998) 706–709.
- [37] M.A. Rieger, A. Parlesak, B.L. Pool-Zobel, G. Rechkemmer, C. Bode, Diets high in fat and meat, but low in fiber increase the genotoxic potential of fecal water, *Carcinogenesis* 20 (1999) 2317–2326.
- [38] M. Gleis, N. Habermann, K. Osswald, C. Seidel, C. Persin, G. Jahreis, B.L. Pool-Zobel, Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet assay: a biomarker model, *Biomarkers* 10 (2005) 203–217.
- [39] S.B. Raffin, C.H. Woo, K.T. Roost, D.C. Price, R. Schmid, Intestinal absorption of hemoglobin iron-heme cleavage by

- mucosal heme oxygenase, *J. Clin. Invest.* 54 (1974) 1344–1352.
- [40] A.T. McKie, D. Barrow, G.O. Latunde-Dada, A. Rolfs, G. Sager, E. Mudaly, M. Mudaly, C. richardson, D. Barlow, A. Bromford, T.J. Peters, K.B. Raja, S. Shirali, M.A. Hediger, F. Farzaneh, R.J. Simpson, An iron-regulated ferric reductase associated with the absorption of dietary iron, *Science* 291 (2001) 1755–1759.
- [41] H. Gunshin, B. Mackenzie, U. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a mammalian proton-coupled metal-ion transporter, *Nature* 388 (1997) 482–488.
- [42] N. Haag, Modulation von Zellwachstum und Genexpression durch Eisen und Butyrat in humanen Kolonzelllinien. Thesis 2003. Friedrich-Schiller-University, Jena.