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New Marker of Colon Cancer Risk Associated with Heme Intake: 1,4-Dihydroxynonane Mercapturic Acid

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Abstract

Background: Red meat consumption is associated with an increased risk of colon cancer. Animal studies show that heme, found in red meat, promotes preneoplastic lesions in the colon, probably due to the oxidative properties of this compound. End products of lipid peroxidation, such as 4-hydroxynonenal metabolites or 8-*iso*-prostaglandin-F₂α (8-*iso*-PGF₂α), could reflect this oxidative process and could be used as biomarkers of colon cancer risk associated with heme intake.

Methods: We measured urinary excretion of 8-*iso*-PGF₂α and 1,4-dihydroxynonane mercapturic acid (DHN-MA), the major urinary metabolite of 4-hydroxynonenal, in three studies. In a short-term and a carcinogenesis long-term animal study, we fed rats four different diets (control, chicken, beef, and blood sausage as a high heme diet). In a randomized crossover human study, four different diets were fed (a 60 g/d red meat

baseline diet, 120 g/d red meat, baseline diet supplemented with heme iron, and baseline diet supplemented with non-heme iron).

Results: DHN-MA excretion increased dramatically in rats fed high heme diets, and the excretion paralleled the number of preneoplastic lesions in azoxymethane initiated rats ($P < 0.0001$). In the human study, the heme supplemented diet resulted in a 2-fold increase in DHN-MA ($P < 0.001$). Urinary 8-*iso*-PGF₂α increased moderately in rats fed a high heme diet ($P < 0.0001$), but not in humans.

Conclusion: Urinary DHN-MA is a useful noninvasive biomarker for determining the risk of preneoplastic lesions associated with heme iron consumption and should be further investigated as a potential biomarker of colon cancer risk. (Cancer Epidemiol Biomarkers Prev 2006;15(11):2274–9)

Introduction

Red meat consumption is associated with an increased risk of colon cancer (1). The mechanism linking red meat to colon cancer is not yet clear, but red meat is the largest source of heme, which has independently been linked to colon cancer risk in women who consumed blood pudding twice a month compared with nonconsumers (2). These epidemiologic observations are in line with animal studies: dietary heme, in the form of hemoglobin or red meat, promotes putative precancerous lesions, aberrant crypt foci, and mucin-depleted foci (MDF) in the colon of rats given a carcinogen injection and fed a low-calcium diet (3, 4). Heme was previously shown to stimulate production of endogenous *N*-nitroso compounds, which can account for the increased risk of colorectal cancer observed in epidemiologic studies (5). Furthermore, the heme-induced promotion is associated with increased concentration of lipoperoxides in fecal water, involving lipid peroxidation as another mode of activity of heme. Lipid peroxidation yields end products, such as alkanes, aldehydes, and isoprostanes and a major end-product is 4-hydroxynonenal. 1,4-Dihydroxynonane mercapturic acid (DHN-MA), previously shown to be the major urinary metabolite of 4-hydroxynonenal (6), could represent a specific and noninvasive biomarker of the lipid peroxidation process (7). As heme intake, colon carcinogenesis, and high luminal peroxidation are linked, we analyzed urinary DHN-MA and 8-*iso*-prostaglandin-F₂α (8-*iso*-PGF₂α) excretion, the latter being a widely used lipid peroxidation biomarker (8),

in three separate studies. These studies included two animal studies, both involving four different diets: (a) a long-term carcinogenesis study to determine the link between urinary DHN-MA and carcinogenesis in rats with chemically induced cancer and (b) a short-term nutritional study to determine the association between diet and DHN-MA urinary excretion. In addition, a third study used samples from a previous human study, which showed that heme but not inorganic iron or protein increased endogenous *N*-nitrosation in the human colon (5), to see whether volunteers excrete more DHN-MA after consuming a diet rich in heme compared with a low-heme diet.

Materials and Methods

Animal Studies. Fischer 344 female rats were purchased at 4 weeks of age from Iffa Credo (St Germain l'Arbresle, France). The rats were distributed randomly in pairs (long-term carcinogenesis protocol) or individually (short-term nutritional protocol) into stainless steel wire-bottomed cages. Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. All animals were allowed 7 days of acclimatization to the room, their cages, and control diet. Analyses were done on blinded duplicate samples.

Long-term Carcinogenesis Protocol. Animals were divided into four groups, and each group consisted of five rats. All rodents were i.p. injected with the carcinogen azoxymethane (Sigma Chimie, St. Quentin-Fallavier, France; 20 mg/kg of body weight) in NaCl (9 g/L). Seven days after the injection, the rats were allowed free access to their respective diet (control, chicken, beef, and blood sausage diets described below) for a duration of 100 days. Feed was changed every second or third day and water once a week. Urine samples

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Table 1. Composition of animal diets

	Control	Chicken	Beef	Blood sausage
Chicken	0	600	0	0
Beef	0	0	600	0
Blood sausage	0	0	0	600
Lard	150	122	40	112
Safflower oil	50	50	50	50
Casein*	500	1.1	48.5	115
Corn starch	60	60	60	5
Sucrose	139.5	68	102	20
Cellulose	50	50	50	50
L-Methionine	3	3	3	3
Mineral mix [†]	35	35	35	35
Vitamin mix	10	10	10	10
CaHPO ₄	2.7	1.4	1.6	1.8
Ferric citrate	0.45	0.35	0	0

NOTE: Values are g/kg of diet.

*Low-calcium casein.

[†]AIN-76 mix, but 500g/kg of dibasic calcium phosphate replaced by sucrose in mineral mix.

were collected on day 77 and stored at -20°C . Rats were sacrificed at day 100, and MDF were scored blindly as already reported (3, 4). Colons, after fixation in 10% formalin, were stained overnight with high-iron diamine-Alcian blue procedure to evaluate mucin production (4). After rinsing, colons were counterstained in 1% Alcian blue solution for 30 minutes. MDF number and number of crypts per MDF were scored blindly under light microscope at $\times 40$ magnification.

Short-term Nutritional Protocol. During the experiment, rats (five rats per group) in this study were fed the four diets (control, chicken, beef, and blood sausage diets) described below *ad libitum* and had free access to tap water. Urine was

collected daily in tubes containing 500 μL of 360 mmol/L butylated hydroxy-toluene, labeled with blind codes, and stored at -20°C . Animals were sacrificed 15 days after the beginning of the experiment.

Experimental Diets. The diets used for the short-term and long-term animal studies were based on a modified AIN-76 diet in a powdered form (Table 1). All diets were low calcium because they contained dibasic calcium phosphate at 2.7 g/kg. Three meat diets given to three groups of rats were formulated to contain varying concentrations of heme as hemoglobin or myoglobin by the addition of freeze-dried beef, chicken, or blood sausage at 60% meat of the total diet, whereas the control diet contained only casein as protein source. The beef diet contained 222 $\mu\text{g/g}$ of heme, whereas none was detected in the chicken diet. The low-fat blood sausage diet contained 5.9 mg/g of heme. All diets were balanced for protein (50%), fat (20%), calcium (0.8 g/kg), and iron (0.14 g/kg) by addition of casein, lard, safflower oil, calcium phosphate, and ferric citrate. However, blood sausage is overloaded with heme, and the blood sausage diet could not be balanced for iron (0.95 g/kg). The diets were made up fortnightly and maintained at -20°C to avoid lipid peroxidation.

Human Intervention. Eight healthy male volunteers (age ranging between 24 and 74 years) were randomly assigned to each of four 15-day dietary periods. As previously described (5, 9), volunteers lived in a metabolic suite where all food and drink was provided, and all specimens were collected. The Cambridge Local Research Ethics Committee gave permission for the study and each volunteer signed a consent form after receiving a detailed explanation of the study protocol and aims. All diets were constant in fat and dietary fiber. The four dietary periods were a 60 g/d red meat diet (containing 9.9 mg/d iron), which constitutes the baseline diet (Table 2);

Table 2. Composition of the human baseline diet

Day 1	Day 2	Day 3
Breakfast Orange juice (100 g) Weetabix (20 g) White bread (50 g) Low fat spread (20 g) Marmalade (20 g) Semi-skimmed milk (300 g) Lunch White bread (130 g) Mayonnaise (33 g) Beef (20 g) Ketchup (25 g) Tomato (50 g) Biscuit (17 g) Apple (150 g) Dinner Lasagna: Beef mince (<5% fat; 40 g) Lasagne (raw wt; 50 g) Onion (75 g) Tomato (75 g) Juice (25 g) Mixed herbs (2 teaspoons) Stock (150 g) Corn flour (5 g) Plain flour (15 g) Butter (15 g) Cheddar cheese (35 g) Skimmed milk (150 g) Canned peaches (150 g) Syrup (50 g) Vanilla ice cream (30 g) Polycal (120 g)	Orange juice (100 g) Weetabix (20 g) White bread (50 g) Low fat spread (20 g) Marmalade (20 g) Semi-skimmed milk (300 g) White bread (130 g) Mayonnaise (33 g) Beef (20 g) Relish (25 g) Tomato (50 g) Biscuit (17 g) Apple (150 g) Sweet and sour pork: Lean pork (<7% fat; 40 g) Green pepper (35 g) Red pepper (40 g) Onion (75 g) Garlic (3 g) Ginger (3 g) Worcester sauce (5 g) Sugar (10 g) Corn flour (5 g) Stock (150 g) Butter (20 g) White rice (raw wt; 50 g) Canned pears (150 g) Syrup (50 g) Vanilla ice cream (60 g) Polycal (170 g)	Orange juice (100 g) Weetabix (20 g) White bread (50 g) Low fat spread (20 g) Marmalade (20 g) Semi-skimmed milk (300 g) White bread (130 g) Mayonnaise (33 g) Beef (20 g) Pickles (25 g) Tomato (50 g) Biscuit (17 g) Orange with peel (300 g) Cottage pie: Beef mince (<5% fat; 40 g) Canned sweetcorn (100 g) Potato (150 g) Green beans (50 g) Onion (75 g) Stock (150 g) Mixed herbs (2 teaspoons) Butter (20 g) Corn flour (5 g) Canned apricots (150 g) Syrup (50 g) Vanilla ice cream (60 g) Polycal (195 g)

NOTE: All meat weight given as cooked weight. For the 120 g/d red meat diet, beef at lunch was 40 g instead of 20 g, and meat at dinner was 80 g instead of 40 g with a reduction of polycal (80, 130, and 160 g for days 1, 2, and 3, respectively).

Table 3. Red meat intake increases 4-hydroxynonenal in diet and urinary DNH-MA excretion in rats treated with a carcinogen (long-term protocol, five rats per group)

	Iron in diet (g/kg)	Heme in diet (g/kg)	HNE in diet (mg/kg)	Urinary DNH-MA (ng/24 hours), mean (95% CI)	Urinary 8- <i>iso</i> -PGF ₂ α (ng/24 hours), mean (95% CI)
Control diet	0.14	ND	0.14	128 (103-152)	11.1 (7.9-14.3)
Chicken diet	0.14	ND	0.79	300 (228-371)	11.7 (9.6-13.9)
Beef meat diet	0.14	0.22	3.3*	588 (285-891)*	13.2 (8.3-18.1)
Blood sausage diet	0.95	5.9	66.4 ^{†,‡}	9,439 (8,094-10,782) ^{†,‡}	26.1 (18.6-33.5) [†]

Abbreviations: ND, Not detectable; HNE, 4-hydroxynonenal; 95% CI, 95% confidence interval.

*Significantly different from control diet, after excluding blood sausage diet results from the analysis ($P < 0.01$, Dunnett's test).

[†]Significantly different from control diet ($P < 0.0001$, Dunnett's test).

[‡]Significantly different from beef diet ($P < 0.01$, Dunnett's test).

a 120 g/d red meat diet (containing 12.5 mg/d iron); a 60 g/d red meat diet supplemented with heme iron in the form of 50 g/d of liver pâté on menu days 1 and 3; 70 g/d of blood sausage on menu of day 2 of a 3-day rotating menu (total iron content: 17.7 mg/d); a 60 g/d red meat diet supplemented with 300 mg/d ferrous gluconate tablet (35 mg of ferrous iron; total iron content: 44.9 mg/d). Those diets were made up of normal, palatable foods to total 10 MJ/d. Volunteers with a higher energy requirement than the 10 MJ baseline diet were given 1 MJ increments of white bread (50 g), low-fat spread (20 g), or marmalade (20 g). Dietary intakes of each volunteer varied from 10 to 12 MJ/d. Three consecutive 24-hour urine collections were made for 3 days at the end of each dietary period (days 13, 14, and 15 corresponding to menu days 1, 2, and 3) and stored at -20°C . The blood sausage and pâté were analyzed for 4-hydroxynonenal (see below).

Dietary Heme Assay. The amount of heme in rat diets, liver pâté, and blood sausage was determined according to Van den Berg et al. (10). Briefly, an acidified methanol-chloroform extract (final concentration of HCl was 1 mol/L) was taken from 20 mg of meat. After centrifugation, the chloroform phase was recovered and dried under nitrogen. Samples were dissolved in 0.45 mL of KOH (250 mmol/L), sonicated for 5 minutes (Cleanet, Hans Grieshaber, Switzerland), and mixed with 0.45 mL of distilled water, 3.75 mL of 2-propanol, and 0.75 mL of HCl (1.15 mol/L). This mix was homogenized and then centrifuged for 10 minutes at $1,500 \times g$, and the supernatants were assayed for their heme content. Supernatants (50 μL) were mixed with 1 mL of glacial acetic acid. Subsequently, 50 μL of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.12 mol/L freshly prepared) and HCl (4.5 mol/L) were added. Samples were immediately mixed and incubated at 60°C for 30 minutes. Two milliliters of 2-propanol/water (1:1, v/v) were added before fluorescence measurement using excitation and emission wavelengths of 400 and 594 nm (JY3D, Jobin-Yvon, France). Blanks were obtained with the same protocol but without the incubation at 60°C .

Dietary 4-Hydroxynonenal Assay. Meat or diet samples (1.5-15 g depending on the dryness of the sample) were homogenized in water (10 mL) and centrifuged twice. The supernatants were pooled and extracted twice with methylene chloride. After evaporation under vacuum, the residue was defatted using isooctane/acetonitrile (1:4, v/v) and then dissolved in phase A of the high-performance liquid chromatography system used for analysis. High-performance liquid chromatography system (Hewlett-Packard series 1100 with low-pressure quaternary gradient; Hewlett Packard, Labège, France) was equipped with a Spherisorb ODS2 reverse-phase column (25 \times 4.6 mm, 5 μm) and a 100- μL loop, and the oven was set at 35°C . UV detection was set at 221 nm, and the following elution gradient was used: 100% A for 12 minutes then 100% A to 100% B from 12 to 18 minutes and then 100% B from 18 to 25 minutes, where A was water/acetonitrile (65:35,

v/v) and B was water/acetonitrile (45:55, v/v), at a flow rate of 1 mL/min. Retention time of 4-hydroxynonenal was 9.2 minutes. 4-Hydroxynonenal quantitation was done by using a calibration curve. To calculate the recovery of 4-hydroxynonenal in the samples, tritiated 4-hydroxynonenal was added before the extraction, and radioactivity was analyzed by radio-high-performance liquid chromatography. Extraction recoveries were used to calculate free and total 4-hydroxynonenal.

Urinary DNH-MA and 8-*Iso*-PGF₂α Assay. DNH-MA and 8-*iso*-PGF₂α measurements in urine were achieved using competitive enzyme immunoassay as previously described (11-13), using DNH-MA-linked acetylcholinesterase enzyme or 8-*iso*-PGF₂α-linked acetylcholinesterase as tracer, respectively. All samples were analyzed blindly, and the code was broken only after data had been fully processed and dispatched to all authors. The urine samples were assayed in duplicates.

Statistical Analysis. Results were analyzed using Systat 10 for Windows and GraphPad Prism softwares and given as means with 95% confidence intervals. Values were considered first using a one-way ANOVA. If a significant difference was found between groups (bilateral $P < 0.05$), then each experimental group was compared with the control group using multiple comparison test. The Dunnett's test was used to compare each group of rats to the control group, and the Student-Newman-Keuls pairwise comparison was used to compare dietary periods in the human study. Because the blood sausage diet induced a 100-fold increase in DNH-MA excretion in rats, variance values for this group obscured the comparisons with other groups. Thus, a second ANOVA analysis of the data from the animal studies was done after excluding value from the blood sausage group, as explained in

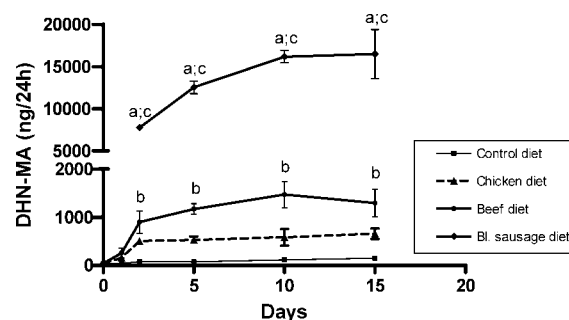


Figure 1. Blood sausage and beef meat diets and DNH-MA excretion in rat urine (short-term protocol, five rats per group). a, $P < 0.0001$, significantly different from control (Dunnett's test); b, $P < 0.01$, significantly different from control, after excluding blood sausage results from the analysis (Dunnett's test); c, $P < 0.01$, significantly different from beef (Dunnett's test).

Table 4. Effect of heme and 4-hydroxynonenal content of the diet on urinary DHN-MA in human volunteers

Diet	Heme (mg/d)	Iron (mg/d)	HNE (μ g/d)	Urinary DHN-MA (ng/24 hours), mean (95% CI)	Urinary 8-iso-PGF $_{2\alpha}$ (ng/24 hours), mean (95% CI)
Low red meat (60 g/d)	55	9.9	ND	1,719 (1,558-1,880)	949 (745-1,155)
120 g/d red meat	110	11.2	ND	1,974 (1,744-2,204)	961 (805-1,118)
Low red meat supplemented with heme iron as liver pâté (50 g) or as blood sausage (70 g)	80	17.7	5.5 (pâté)	1,957 (1,587-2,328)	959 (660-1,257)
Low red meat diet supplemented with inorganic iron	205	17.7	875	4,147 (1,976-6,317)*	906 (612-1,201)
	55	44.9	ND	1,726 (1,544-1,907)	898 (745-1,053)

Abbreviations: ND, Not detectable; HNE, 4-hydroxynonenal; 95% CI, 95% confidence interval.

*Significantly different from all other groups (all $P < 0.001$, Student-Newman-Keuls test on all data; all $P < 0.001$, same test on paired data from six volunteers).

a note to Table 3. Human data were also analyzed a second time, by repeated-measure ANOVA. This second analysis of paired data yielded the same P , but it seemed more appropriate than the unpaired ANOVA because matching was effective ($P = 0.026$), and it eliminated problems of unequal variances.

To evaluate the relationship between histologic (MDF) and dietary (iron, heme, and 4-hydroxynonenal) or urinary (DHN-MA and 8-iso-PGF $_{2\alpha}$) variables, Pearson correlation coefficients were calculated. Only correlations with $P < 0.01$ were considered statistically significant (Bonferroni post-test).

Results

DHN-MA Excretion in Rats. The blood sausage diet increased DHN-MA excretion by 73-fold ($P < 0.0001$) in urine collected after 77 days on the diet compared with urine collected from rats on the control diet (Table 3). The beef diet increased DHN-MA by 4.6-fold ($P < 0.01$), but the chicken diet did not significantly change DHN-MA excretion, compared with the control diet. We observed a strong link between heme intake and DHN-MA excretion ($P < 10^{-10}$).

In the short-term study, similar modulations of DHN-MA excretion were observed in the urine of rats not given a carcinogen (Fig. 1). For instance, after 15 days on a blood sausage diet, DHN-MA excretion increased 113-fold compared with the control diet. The increase was fast, because 24 hours after the start of experimental diets, DHN-MA excretion had already increased 50-fold in non-initiated rats fed the blood sausage diet. A plateau was reached within 5 to 10 days on all diets.

DHN-MA Excretion in Human Volunteers. A high-heme diet increased urinary DHN-MA in comparison with the other diets (all $P < 0.001$; Table 4). In contrast, no difference in DHN-MA excretion was observed between periods where volunteers were given 60 g/d red meat, 120 g/d, or inorganic iron (all $P > 0.05$; Table 4).

To elucidate this difference in DHN-MA excretion, we analyzed the heme and 4-hydroxynonenal content of meat used in the human intervention. Red meat contained 0.92 mg of heme per gram of meat; blood sausage contained 2.15 mg but pâté only 0.49 mg per gram of meat (Table 4). Thus, as in the rat studies, we observed a significant correlation between heme intake and DHN-MA excretion ($r = 0.94$, $P < 0.05$, $n = 5$). The amount of 4-hydroxynonenal in liver pâté was 0.11 ppm and 12.5 ppm in blood sausage, whereas it was not detectable in beef.

8-Iso-PGF $_{2\alpha}$ Excretion in Rodents and in Human Volunteers. The blood sausage diet increased significantly 8-iso-PGF $_{2\alpha}$ excretion in both rat studies ($P < 0.0001$; Table 3; Fig. 2). In contrast with DHN-MA excretion, 8-iso-PGF $_{2\alpha}$ did not increase when the rats were fed the beef diet (Table 3; Fig. 2). In the human protocol, no modification of this marker was observed for any of the diets (Table 4).

MDF Promotion in Long-term Carcinogenesis Protocol.

Beef- and blood sausage-fed rats had more MDF than control rats ($P < 0.01$), and promotion by blood sausage was more potent than promotion by beef ($P < 0.05$). The chicken-based diet, the low-heme diet, did not promote MDF formation (Fig. 3). As shown in Fig. 3, MDF promotion increased with DHN-MA values in the urine of rats treated with a carcinogen ($P < 9 \times 10^{-5}$). Among the tested variables urinary DHN-MA and 8-iso-PGF $_{2\alpha}$ (dietary 4-hydroxynonenal, heme, and iron), DHN-MA and 4-hydroxynonenal were the best candidate variables correlated with MDF variation (Pearson coefficient: 0.654 and 0.577, respectively; $P < 0.001$).

Discussion

These results show that intake of a meat-based diet containing heme increases the urinary excretion of DHN-MA in both animal models and in humans. In rats given blood sausage, the increase in DHN-MA was strikingly high, and concentrations reached a level never seen previously in any urine sample (7). The effect of diet was very fast because the DHN-MA value increased by a 50-fold factor after only 24 hours on the blood sausage diet. A 5- to 9-fold increase was also seen in rats given a beef meat diet. In human volunteers, the 2-fold DHN-MA increase on the high-heme diet was very significant but much less striking than in rats.

Red meat promotes colon carcinogenesis in rodents, and this promotion is linked with heme intake (3, 4). In a previous study, we have shown that heme-rich meat diets increase the number of MDF, preneoplastic lesions in the colon of carcinogen-initiated rats. Promotion is significantly greater in rats given a high-heme blood sausage diet than a medium-heme beef diet. A low-heme chicken diet does not promote MDF. This promotion is associated with lipid peroxidation in the fecal water, assessed by the thiobarbituric acid reactive

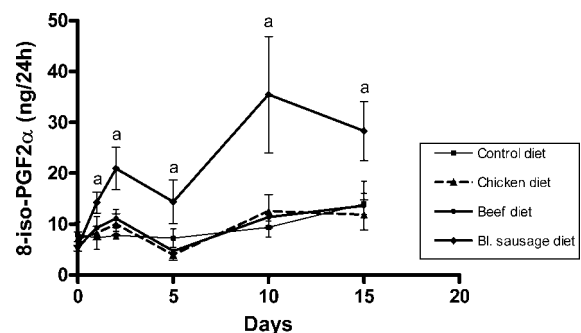


Figure 2. Blood sausage diet and 8-iso-PGF $_{2\alpha}$ excretion in rats urine (short-term protocol, five rats per group) a, $P < 0.0001$, significantly different from control (Dunnett's test).

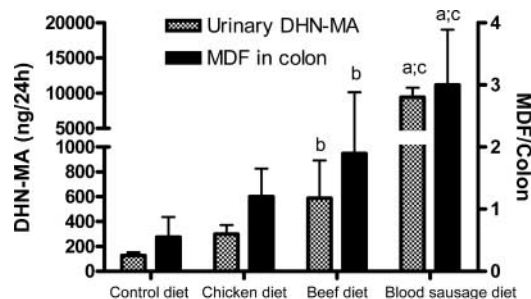


Figure 3. Association between urinary DHN-MA and MDF in rats (five rats per group). a, $P < 0.0001$, significantly different from control (Dunnett's test); b, $P < 0.01$, significantly different from control, after excluding blood sausage results from the analysis (Dunnett's test); c, $P < 0.01$, significantly different from beef (Dunnett's test). MDF data were published in ref. 4.

substance assay (3, 4). 4-Hydroxynonenal is considered as a reliable biomarker of lipid peroxidation, and it is also a cytotoxic and a genotoxic agent, a real "second messenger of oxidative stress," via alteration(s) of cellular functions and the formation of exocyclic DNA adducts (14). 4-Hydroxynonenal is also considered as a cell signaling molecule (15). However, 4-hydroxynonenal is not excreted into urine and thus cannot be directly measured from noninvasive sampling techniques, although DHN-MA, the major urinary metabolite of 4-hydroxynonenal, bears the same advantages without this drawback.

This DHN-MA excretion was related to heme intake (Table 3). Heme induces lipid peroxidation in the diet and the formation of 4-hydroxynonenal, which is the precursor of DHN-MA, but the same phenomenon also likely occurs during digestion. DHN-MA reflects then both lipid peroxidation occurring in the diet and during digestion. Its determination gives a more complete view of these oxidative events than dietary 4-hydroxynonenal. DHN-MA was not a consequence of MDF development, but it was on the contrary related to MDF promotion (Fig. 3). Indeed, similar DHN-MA levels were seen in the urine of rats pretreated with a carcinogen or not, and DHN-MA increased quickly after the blood sausage diet was given.

Human volunteers given a high-heme diet excreted twice the amount of DHN-MA than when they were on a lower-heme diet. The fact that the effect was much smaller in humans than in rats could be explained by differences in heme doses, and in dietary protective agents: (a) the daily intake of heme was 60 times higher in rats than in humans, when estimated by kg of metabolic weight; (b) the diet given to human controls contained 60 g/d of beef meat, whereas the diet for control rats contained no meat and no heme; (c) rat diets were designed to contain very small amount of heme-blocking calcium, whereas human diet contained normal amounts of calcium (9); (d) rat diets were designed to contain 5% safflower oil, this oil is low in vitamin E and contains 70% of linoleic acid, which get easily peroxidized and yields 4-hydroxynonenal; (e) rat diets were made of purified components, free of antioxidant agents, whereas the human diets contained fruits and vegetables (Table 2), and possibly antioxidant additives like butylated hydroxyanisole, which inhibits both peroxidation and carcinogenesis (3, 4).

Preneoplastic lesions and DHN-MA excretion are clearly associated with dietary heme iron, but not with inorganic (non-heme) iron. The link between DHN-MA and carcinogenesis is likely to be related to lipid peroxidation and production of 4-hydroxynonenal, which is the precursor of DHN-MA. We show here that 4-hydroxynonenal concentration in diets was related to the concentration of heme-iron and not of inorganic

iron. Why was 4-hydroxynonenal formed when heme-iron, but not non-heme-iron was present in the diet? 4-Hydroxynonenal is a peroxidation product formed during the oxidation of *n*-6 fatty acids, particularly linoleic acid, which is abundant in foods. 4-Hydroxynonenal links covalently with cysteine, histidine, and lysine (16, 17). Hemoglobin (in blood sausage) and myoglobin (in beef meat) are rich in histidine. Interestingly, 4-hydroxynonenal links covalently with the histidine residues that coordinate iron in heme proteins (18). This covalent link can affect the redox stability of the heme proteins by increasing their oxidation status (19). Thus, 4-hydroxynonenal increases the formation of oxidized metmyoglobin and decreases the ability of metmyoglobin to be enzymatically reduced, therefore enhancing its pro-oxidant activity. One can hypothesize that this increased pro-oxidant activity leads to the peroxidation of the dietary fatty acids and the new generation of 4-hydroxynonenal, which in turn makes covalent links to the heme proteins, increasing the phenomenon. DHN-MA comes from the metabolization of 4-hydroxynonenal in the body (6). Although 4-hydroxynonenal metabolism after oral administration has not been studied yet, one can assume that this rather lipophilic compound can easily go through the intestinal barrier and be metabolized within the body into DHN-MA, among other metabolites, and be eliminated into urine (6).

In contrast with 4-hydroxynonenal, 8-*iso*-PGF₂α originates exclusively from arachidonic acid, which is not usually abundant in foods, except in poultry. 8-*iso*-PGF₂α level, thus, reflects inflammation-induced lipid peroxidation within the body membranes, and this biomarker does not depend on the diet (20). We showed here that a blood sausage diet increased the urinary excretion of 8-*iso*-PGF₂α in rats. This suggests that this diet can quickly induce an inflammatory process, possibly in the gastrointestinal tract. However, this lipid peroxidation biomarker was not sensitive enough to reflect the putative inflammation induced by beef meat in rats or by blood sausage in humans.

Lipid peroxidation in food, and possibly in the gastrointestinal tract, results from the concomitant presence of peroxidizable fatty acids and a pro-oxidant compound and can be modified by the presence of antioxidant compounds. The interest of DHN-MA as a biomarker of risk when compared with the measurement of dietary heme in food or body iron stores is that this compound indeed reflects this food lipid peroxidation process and not only the presence of the pro-oxidant compound. In rats, DHN-MA increase was linked with the promotion of an early biomarker of carcinogenesis. Urinary DHN-MA level increased fast after heme ingestion. Its return to baseline when turning back to a no-heme diet was not looked for here, but DHN-MA seems a short-term risk biomarker. Long-term exposure to heme-rich diets should be better estimated by measuring adducts to protein or DNA in colonic exfoliated cells (21), and 4-hydroxynonenal adducts could be good candidates. Because urine is much easier to collect than exfoliated cells, urinary DHN-MA could be used as short-term biomarker of cancer risk associated with meat-based diets in nutritional studies.

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