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Differential gene expression in rat colon by dietary heme and calcium

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Dietary heme and calcium are alleged modulators of colon cancer risk. Little is known about the molecular and cellular changes in the colon epithelium that are induced by consumption of these unabsorbed nutrients. In this nutrigenomics study, we fed rats high- and low-calcium diets with or without heme. In agreement with previous studies, we found that dietary heme increased the cytotoxicity of fecal water in the colon and elevated epithelial proliferation, a risk factor in colon carcinogenesis. Calcium reduced cytotoxicity and inhibits heme-induced effects. Among 365 colon-expressed genes, we could identify 10 diet-modulated genes that show >2-fold altered expression, of which several are related to colon cell turnover and disease. Mucosal pentraxin (*Mptx*) was the strongest differentially expressed gene, ~10-fold down-regulated by dietary heme and 3-fold up-regulated by calcium. cDNA microarray and quantitative PCR analysis show that calcium significantly inhibits the effects of heme, which correlates with the physiological effects. Our results indicate that *Mptx* expression is related to colonic cell turnover, and that *Mptx* might be a marker for diet-modulated mucosal integrity. We also show that *Mptx* expression is restricted to the intestine, and occurs predominantly in the colon.

Introduction

The incidence of colon cancer is strongly associated with dietary habits (1). From numerous epidemiological studies it has become clear that the consumption of a typical Western style diet, which is characterized by a high intake of meat and fat, and a low intake of fibre and vegetables, is associated with an increased colon cancer risk (2). According to the model of Kinzler and Vogelstein (3), dietary factors, which lead to colorectal cancer are luminal irritants, which damage epithelial cells and increase tissue regeneration, thereby increasing cell turnover and concomitant the risk for DNA mutations. Long-term accumulation of diet-induced genetic changes underlies the transformation of epithelium to malignant neoplasms.

Abbreviations: *Aldoa*, aldolase A; *CA1*, carbonic anhydrase I gene; CRP, C-reactive protein; *Krt21*, cytokeratin 21; *Mptx*, mucosal pentraxin; *Fxyd4*, FXYD ion transport regulator 4; PCA, principal component analysis; SAP, serum amyloid P component.

In our previous studies we have demonstrated that the association between a high intake of red meat and colon cancer risk might be caused by the high content of heme in red meat (4). In a strictly controlled rat study we found that the intake of a high amount of dietary heme in combination with a 'Westernized diet' (high fat), results in a drastic increase in cytotoxicity of the colonic content and hyperproliferation of the colonic mucosa, which is a risk factor in carcinogenesis (5).

Recently, we have performed *in vivo* expression profiling studies to identify genes that are modulated in the rat colon by dietary heme (6). A microarray containing 2300 rat colon-derived cDNAs as well as commercial genechips (Affymetrix rat U34A, Clontech rat Atlas 1.0) were used to identify heme-modulated genes. Among 10 000 genes, one strongly regulated, pentraxin-like gene was detected. This was a new gene that we named Mucosal pentraxin, *Mptx* (accession no. AY426671). Quantitative PCR confirmed that *Mptx* mRNA levels were 10-fold down-regulated in response to dietary heme *in vivo*, an exceptionally large effect in *in vivo* nutritional gene regulation studies.

To examine our hypothesis that *Mptx* is a potential marker of diet-induced stress of colonic mucosa, and to identify other candidate molecular markers, in the present study we have analyzed gene expression changes of several hundred colon-specific genes in response to dietary calcium, in diets with or without heme. Dietary calcium is commonly regarded as a protective agent in colon carcinogenesis and has beneficial effects on colon health (7,8). Previously, we have shown that the physiological effects of calcium on colonic mucosa are opposite to heme. Moreover, calcium strongly inhibits the heme-induced cytolytic activity of rat fecal water and colonic epithelial hyperproliferation (7).

Our results reveal a set of diet-modulated genes, of which several have been reported to play a role in colon cell turnover or colorectal cancer. We show that calcium induces up-regulation of *Mptx* gene expression and inhibits the heme-induced down-regulation, which correlates with physiological effects of heme and calcium. In addition, we demonstrate that *Mptx* is expressed predominantly in the colon.

Materials and methods

Animals and diets

Wistar rats (outbred, male, 9-week-old, Harlan Horst/Wu, mean body weight 218 g) were housed individually and fed a humanized AIN-93 (9) rodent diet, as described previously (6), differing only in heme and calcium ($CaHPO_4 \cdot 2H_2O$; Fluka) content. Four groups of 16 rats were used: (i) control (20 mmol Ca/kg diet); (ii) heme (Sigma, US) (20 mmol Ca/kg diet and 0.5 mmol heme/kg diet); (iii) calcium (100 mmol Ca/kg diet; and (iv) heme + calcium (100 mmol Ca/kg diet and 0.5 mmol heme/kg diet). Rats were acclimatized for 7 days prior to the start of the feeding experiment, which then lasted for 14 days. Feces were collected daily during days 11–14 of the experiment and were frozen at −20°C. After 2 weeks, eight rats of each group were randomly selected and used to determine *in vivo* colonic cell proliferation (4). On the same day, the other eight rats of each group were killed; the colon was excised, rinsed in 154 mM KCl, the major kathion in colon, and scraped to

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recover the mucosa. Scrapings were frozen and stored in liquid N_2 until total RNA extraction. Isolation and quantification of total RNA was performed as described before (6). The animal welfare committee of Wageningen University, Wageningen, The Netherlands approved the experiments.

In vivo colonic cell proliferation and cytotoxicity of fecal water

After the treatment period of 14 days, rats ($n = 8$) were injected i.p. with [methyl-^{3}H]thymidine (Amersham International, UK; sp. act. 25 Ci/mmol; dose 100 μCi/kg body wt) in 154 mM KCl. After 2 h, the rats were killed and incorporation of [methyl-^{3}H]thymidine per μg DNA was determined in the mucosal scrapings as described before (4). Fecal water was prepared and its cytotoxicity quantified as described before (4). In short, 5 or 10 μl of faecal water was mixed with a human erythrocyte suspension and the samples were incubated for 15 min at 37°C. To compare cytotoxicity of the calcium- and the control-group, 40 or 80 μl fecal water was mixed with erythrocytes and incubated for 2 h at 37°C. In each experiment, a standard curve was made by incubating erythrocytes in 154 mM NaCl (0% hemolysis) and in double-distilled water (100% hemolysis). Cytotoxicity was expressed as the area under the hemolytic curve relative to the maximal area (at 100% lysis).

Microarray experiments and data analysis

In order to analyze gene expression levels, purified RNA from the mucosal scrapings were used for the hybridization of previously described self-made cDNA microarrays (6), containing about 2000 rat colon-derived cDNAs. All clones are sequenced and annotated by means of BLAST searches in the EMBL/GenBank. Redundant clones were removed from the dataset, leaving 365 colon-specific genes. Probe labeling, array hybridization, processing and normalization of the data were performed exactly as described previously (6). In short, mRNA from rat colon scrapings was labeled with Cyanine (Cy)5 (Amersham), and mixed with a standard Cy3-labeled reference sample. Hybridization to rat colon-specific microarrays was performed in a volume of 45 μl overnight at 42°C. Arrays were scanned using a ScanArray 3000 (General Scanning). Data normalization and analysis was performed exactly as described before (6). In short, three different steps were applied: (i) signal intensities of all 2304 spots were normalized to a standard reference sample that was co-hybridized (Cy3 labeled) on every array; (ii) signal intensities were normalized to the mean signal intensity of all eight rats of a diet group; (iii) genes that show intensity below 3× background were rejected. As described in our previous work (6), an outlier test (10) was applied to decide whether outlying expression profiles of rats are significant and should be rejected. Expression profiles of six rats differed unreasonably from those of the other members of the group (tested at a 1% significance level) and were rejected. Specifically, it concerns one rat of the control group, two of the heme group, two of the calcium group and one of the heme/calcium group. In total, 365 unique colon genes were used for biplot comparisons between dietary treatments. Differences in expression levels between diet groups were analyzed for statistical significance by two-tailed Student's t-tests (assuming normal distributions and unequal variance) using Microsoft Excel. Principal component analysis (PCA) was performed using the software package GeneMaths version 2.0 (Applied Maths, Belgium). PCA is a mathematical method that reduces the many dimensions of a large dataset to a few dimensions that explain the majority of the variation between the samples. The dataset consisted of the log transformed signal intensity of all unique genes of all individual rat colons after filtering as described above.

Quantitative real-time PCR

Quantitative real-time PCR was performed to measure the Mptx mRNA levels in the colon of heme- and/or calcium-fed rats, and to determine the type of intestinal tissue in which Mptx is expressed. For the latter experiment, the jejunum, duodenum, ileum and colon of a rat fed a control diet were scraped to recover the mucosa. In addition, tissue was taken from the stomach, cecum and liver. Samples were homogenized in liquid N_2 and total RNA was isolated and quantified as described before (6). One microgram of purified RNeasy (Qiagen) and DNase-treated total RNA was used for the cDNA synthesis (SuperScript^{TM} Preamplification System for First Strand Synthesis, Life Technologies^{TM}). Real-time PCR was performed with the LightCycler^{TM} (Roche) as described before (6). Each reaction (20 μl) contained 10 μl Quantitect SYBR green PCR mix, 4 mM $MgCl_2$, 5 μl of the cDNA dilution and 0.5 μM of forward and reverse primer. The samples were incubated for 15 min at 95°C (denaturation), followed by 45 amplification cycles (15 s 95°C, 25 s 55°C, 14 s 72°C with a slope of 20°C/s). A negative control without cDNA template was run with every assay. Data were normalized against the housekeeping gene aldolase A (*Aldoa*), whose concentration is unaffected by the used dietary conditions (6). In the experiment to determine the tissue in which Mptx is expressed, normalization was performed with a panel of housekeeping genes, including *Aldoa*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin. Primers sequences: *Mptx* and *Aldoa* (6), *GAPDH* (PCR-Select cDNA Subtraction kit; Clontech), β-actin (NM_031144; forward, pos. 347–369; reverse, pos. 636–662). A standard curve for *Mptx* and each reference gene was generated, using serial dilutions of a reference sample (colon cDNA from three control rats). mRNA levels were determined from the appropriate standard curve. The ratio of mRNA levels between *Mptx* and reference genes was determined. Analysis of all samples was performed in duplicate.

Northern blot analysis

Northern blot analysis was performed using pre-made rat tissue blots (BioChain, Clontech) with 2 or 3 μg poly(A)$^+$ RNA, normalized by expression of the β-actin gene, isolated from several different tissues. Blots were hybridized with the full-length *Mptx* sequence (764 bp), radioactively labeled by random priming with [^{32}P]dCTP (Megaprime DNA labelling, Amersham). Hybridization was performed according to the protocol of the supplier.

Results

Colonic effects of dietary heme and calcium

Rats from the controlled heme- and calcium-feeding experiment, showed the expected physiological responses. Softening of feces was observed with heme-fed rats, which may reflect some disturbance of the absorption or secretion function of the colon. Feces from the calcium-fed group were similar to that of the control group. The different dietary treatments had no effect on body weight, food intake and dry weight of fecal output (Table I). The proliferation activity of the colonic epithelium increased almost 2-fold in the heme-fed rats, compared with the control group (Table I). Statistical analysis by Student's t-test showed that the increase of proliferation in the heme group was significant (P-value 0.012).

This heme-induced hyperproliferation was almost completely inhibited by calcium. Colonic cytotoxicity was measured in an erythrocyte lysis assay. The feces of rats fed heme showed extremely high cytotoxicity; 10 μl of fecal water was sufficient to lyse all erythrocytes. When we used 10 μl, the other groups did not differ from the control. An adjusted assay, in which 40 μl of fecal water was added to erythrocytes, was performed with the calcium and control group. The results showed that calcium decreases fecal water cytotoxicity by

Table I. Effects of dietary heme and calcium on Wistar rats ($n = 16$)[a]

	Control	Heme	Calcium	Heme + calcium
Gain in body wt (g/day)	3.8 ± 0.3	3.1 ± 0.4	4.3 ± 0.4	3.1 ± 0.2
Food intake (g/day)	17.1 ± 0.4	17.0 ± 0.6	18.4 ± 0.4	17.7 ± 0.4
Fecal output (g dry weight/day)	0.6 ± 0.03	0.8 ± 0.07	1.0 ± 0.06	1.3 ± 0.07
Proliferation (d.p.m. [^{3}H]thymidine/μg DNA)[c]	62.3 ± 9.3	106.5 ± 10.4[b]	62.0 ± 4.0	71.1 ± 5.6
Cytotoxicity of 5–10 μl fecal water[c] (% lysis)	1.3 ± 0.6	55.9 ± 8.8[b]	0.3 ± 0.1	0.5 ± 0.3
Cytotoxicity of 40–80 μl fecal water[c] (% lysis)	48.8 ± 7.5	–	26.7 ± 1.7[b]	–

[a]Values represent mean ± SEM.
[b]Significantly different from control group according to Student's t-test ($P < 0.05$).
[c]$n = 8$.

Table II. Differential gene expression in response to dietary heme and/or calcium[a] (relative to control)

Gene name	Abbreviation	Accession number	Heme	Calcium	Heme + calcium
Rat mucosal pentraxin	Mptx	AY426671	**−12.5***	**+2.4***	**−3.3***
Rat chloride channel calcium-activated 3	Clca3	NM_017474	**−2.7***	+1.8*	−1.4
Rat FXYD ion transport regulator 4	Fxyd4	NM_022388	**−2.6***	1.0	−1.6
Rat carboxyl ester lipase	CEL	NM_016997	**−2.4***	+1.4	**−2.1***
Mouse intelectin	Itln	NM_010584	**−2.1***	+1.4	−1.7
Mouse DnaJ homolog	Dnajc7	NM_019795	**−2.1***	+1.7	−1.5
Rat carbonic anhydrase 1	CA1	XM_226922	**+3.2***	−1.1	**+2.7***
Rat intestinal fatty acid binding protein	FABP2	NM_013068	**+2.7***	−1.6*	**+4.0***
Rat deleted in malignant brain tumors 1	DMBT1	NM_022849	**+2.5***	−1.1	+1.4*
Rat cytokeratin 21	Krt21	NM_173128	**+2.0***	−1.2	+1.2

[a]The fold change of expression in comparison with the control diet is given and printed in bold where the fold change is >2.
Up-regulation is indicated with a plus sign, while negative values represent down-regulation of the gene.
*Values that are significantly different from the control group according to Student's *t*-test ($P<0.05$).

2-fold compared with the control. The cytotoxicity increase by heme (*P*-value 0.0004) and the decrease by calcium (*P*-value 0.007) were found to be statistically significant in a Student's *t*-test. Taken together, these results are analogous with our earlier work on the colonic effects of dietary heme and calcium (4–7).

Gene expression effects

To analyze gene expression changes in response to the dietary treatments we used cDNA arrays containing 2304 rat colon-derived cDNAs that represent 365 genes in total. Twenty micrograms of total RNA from each individual rat of each group was subjected to the analysis. First, we calculated group average signal intensities, and next, the expression ratio of the treatment group ($n = 6/7$) to the control group ($n = 7$). The genes that showed a differential expression higher than 2.0-fold in response to the dietary treatments are given in Table II, including gene abbreviation, accession number and the fold change in comparison with the control group. A negative value represents down-regulation of the gene, while up-regulation is indicated with a plus sign. Differences between groups were analyzed for statistical significance using Student's *t*-tests ($P < 0.05$). The effects on gene expression were highest in the heme-fed group, which can be expected from the strong effects of heme on the integrity of colonic mucosa. The expression of six genes, including *Mptx*, was down-regulated by >2-fold in response to dietary heme, while four genes were up-regulated. Changes in mRNA levels were less pronounced in the calcium treatment group; an expression effect larger than two was only observed for *Mptx*. For all genes, except FXYD ion transport regulator 4 (*Fxyd4*), the modulation by calcium was opposite to the heme-induced modulation, which correlates with the opposite physiological effects of heme and calcium on colonic mucosa. For these genes, we observed that calcium inhibits the heme-induced expression change, which is also in accordance with the physiological effects. Evidently, these genes reflect diet-induced colonic processes. All the diet-modulated genes in Table II were reported previously to exhibit expression in the intestine of rats or mice.

We also performed principle component analysis, an alternative way to study gene expression profiles, on our data set. The dataset consisted of log-transformed signal intensities of all rats, normalized exactly as described before (6), with previously defined groups. In the two-dimensional plot created by principle component analysis (Figure 1A) the rats are distributed according to the similarity of their expression profile. Clearly, the rats from the heme group are farthest away from the control animals, indicating that the expression patterns of these groups are most different. The calcium-fed animals are located even farther away from the heme-fed rats, on the opposite side of the control rats, indicating opposite genetic effects of heme and calcium. The heme + calcium group is placed in between, as expected. Distribution of rats within one group predominantly reflects biological variation of the animals. Some variation may be due to the technical procedures, such as RNA labeling and hybridization, although the duplo experiments indicate that our microarray experiments are highly reproducible, which has also been shown in our previous study (6).

The contribution of each gene to the discrimination between the different diet groups is shown in Figure 1B. The genes with the largest distance from the centre explain most of the variation between the four groups. Selection of 2% of all genes with the largest or the smallest value on principle component 1 (the *x*-axis) yields a list that includes all genes, except *Fxyd4*, that were also identified by fold-change calculations (Table II).

Validation of diet-induced expression changes of Mptx

The expression of the novel rat gene *Mptx* is most distinct between the different diet groups and we consider this gene as a new potential molecular marker for diet-modulated processes in colon mucosa. To validate the strong effects of heme and calcium on *Mptx* expression that was observed on microarrays, we performed LightCycler-based real-time PCR. Normalization of the samples was performed against the gene *Aldoa*, a housekeeping gene that was not modulated by the different diets, as we observed in the microarray experiments (not shown). Figure 2 shows the relative *Mptx* expression levels of four control animals and five from the different treatment groups, as well as average values. The Q-PCR results confirm the expression changes detected by the array in response to the different dietary treatments, with the same magnitude of the changes (table in Figure 2).

Site of synthesis of rat Mptx

To determine the type of tissue in which *Mptx* is expressed, we used pre-made rat tissue mRNA northern blots, containing 2 or 3 μg of polyA$^+$ RNA per lane from different rat tissues. A ^{32}P-labeled probe, corresponding to the full-length sequence of rat *Mptx* was hybridized to the blots. A positive signal was only

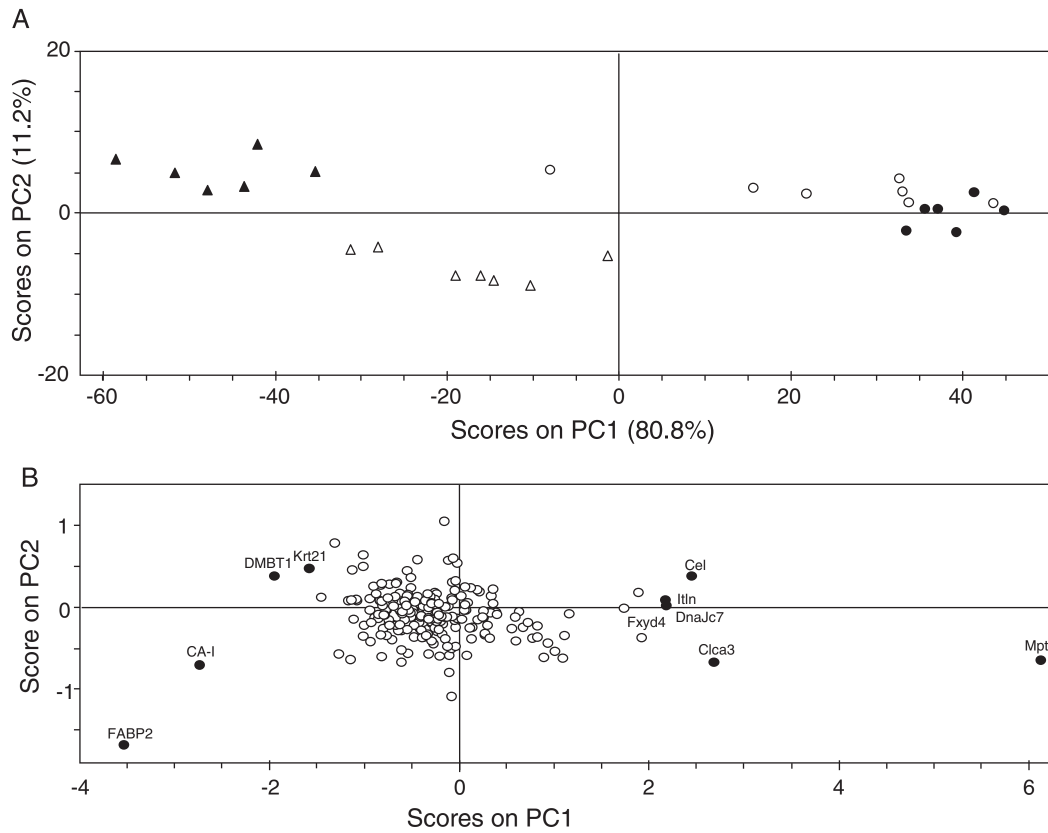

Fig. 1. PCA of microarray data. (**A**) Objects in the plot represent expression profiles from individual rats. The two principal components explaining the majority of the variation in the dataset are plotted. Open circles, control group; closed circles, calcium group; open triangles, heme group; closed triangles, heme + calcium group. The expression profiles of the heme group and the heme + calcium group are clearly distinct from the controls. Rats from the calcium group are more close to the controls, due to small differential gene expression between both groups. (**B**) Dots represent the genes that explain variation between rat expression profiles. The genes with largest distance from the centre contribute most to the variation between the four groups. Black dots represent the 2% of genes with largest distance from centre, including all genes that are selected with a threshold of 2-fold differential expression, with exception of *Fxyd4*.

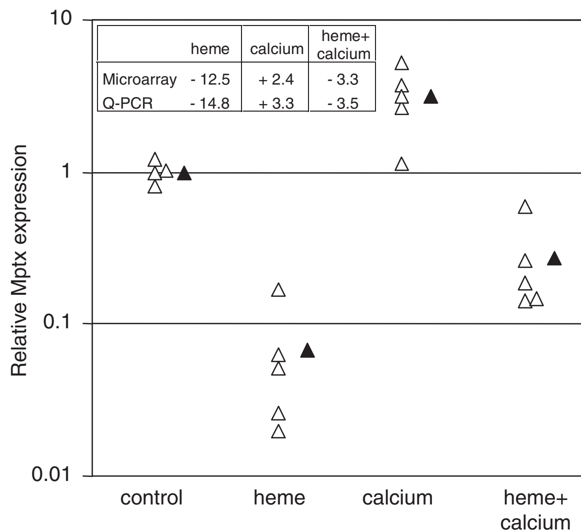

Fig. 2. Relative expression of *Mptx* in rat colon in response to different dietary treatments, measured by quantitative real-time PCR. Expression values represent mRNA levels relative to aldolase A mRNA. The average expression of the control group was set at 1. Open symbols represent individual rats; closed symbols are group averages. The table in the left corner indicates the group average effect of heme and/or calcium on *Mptx* expression measured by microarray and Q-PCR.

Table III. Relative *Mptx* expression levels per μg totRNA measured by real time-PCR

Tissue	*Mptx* level/μg total RNA	No *Mptx* expression
Colon	100%	Spinal cord, prostate,
Cecum	0.06%	thyroid, heart, adrenal gland,
Ileum	0.06%	bladder, brain, kidney,
Jejunum	0.02%	lung, spleen, eye,
Duodenum	0.01%	skeletal muscle, human lung
Stomach	0.12%	bladder, brain, kidney,
Liver	<0.01%	lung, spleen, eye,
Blood	0.01%	skeletal muscle, human lung

Mptx expression in colon was set at 100%. The right column shows all tissues that were negative for *Mptx* expression as determined by northern blotting experiments.

obtained for the small intestine, stomach and colon (not shown). Other tissues tested for expression include rat prostate, thyroid, spinal cord, eye, bladder, adrenal gland, heart, brain, kidney, lung, spleen, skeletal muscle and human lung, and were negative (Table III).

With LightCycler-based real-time PCR, *Mptx* expression levels within distinct regions of the gastrointestinal tract were quantified. For this purpose, we isolated RNA from the rat

stomach, duodenum, jejunum, ileum, cecum and colon. In addition, RNA was isolated from the liver, the site of synthesis of the related serum pentraxins serum amyloid P component (SAP) and C-reactive protein (CRP). When comparing gene expression levels between samples from different tissues, it is difficult to select a valid reference for data normalization. Normalization against cell number is not feasible and the use of a housekeeping gene is not very reliable, since a gene with constant expression levels in these different tissues is not known. Bustin (11) recommended that normalization of *in vivo* tissue samples is preferably carried out against total cellular RNA content, rather than housekeeping genes. In accordance, *Mptx* copy numbers were determined for each tissue in 1 μg of total RNA. The results show that in a rat fed a control diet, *Mptx* is predominantly expressed in the colon (Table III). Alternatively, normalization of *Mptx* RNA levels from the different tissues was performed against a panel of housekeeping genes, including *Aldoa*, *GAPDH* and β-actin. Again, the results showed that *Mptx* expression is highest in the colon.

Discussion

We have studied the expression changes of 365 rat colon-specific genes, including *Mptx*, in response to the intake of dietary calcium and/or heme, both alleged modulators in colon carcinogenesis. We could identify 10 genes that show a >2-fold expression change in response to the dietary components, of which several genes are known to be involved in cell turnover of colonic epithelium. The most prominent effects were observed for *Mptx*, namely 30-fold differential expression. Secondly, we show that the rat *Mptx* is expressed exclusively in the colon, in contrast to related pentraxins, which are liver-synthesized. Taken together, the results establish a potentially important role for *Mptx* as a genetic biomarker for diet-induced stress in colonic mucosa.

The effects of heme and calcium on fecal water cytotoxicity and epithelial cell proliferation corroborate our previous work (4–7). We believe that dietary heme, which is not absorbed in the proximal gastrointestinal tract, forms a cytotoxic complex in the colon that damages epithelial cells and induces compensatory cell proliferation (5). Calcium is commonly regarded as a protective agent in colon carcinogenesis (8). In our rat model system, supplemental dietary calcium inhibited fecal water cytotoxicity (7). Moreover, calcium protected against the detrimental effects of heme, which is probably due to the fact that insoluble, amorphous calcium phosphate precipitates dietary heme and thus prevents the formation of the heme-induced cytotoxic factor (7). It should be realized that the control diet used in this study, is a low-calcium diet, which aims to mimic the deficient daily intake of calcium in humans of 400 mg (10 mmol), assuming a daily dry weight intake of 500 g food. This diet contains less calcium than is optimal for rats and may therefore be considered to be deficient. Our high-calcium diet mimics a human calcium intake of 2000 mg/day. This is at the high end of daily calcium intake in humans and is comparable with the recommended calcium concentration in rat diets (9).

The present study shows the molecular changes in colonic epithelium in response to dietary heme and/or calcium, using rat colon-derived cDNA microarrays generated in a previous experiment (6). In concordance with our previous reported experimental design, we used two replicate arrays for each rat, which enables us to adequately discern experimental variation from labeling and/or hybridization artefacts. To select genes that were most differentially expressed between the diet groups, we used an arbitrary threshold of 2.0-fold or greater expression change, which yielded 10 differentially expressed genes. Expression changes, and the magnitude of these changes were analyzed across all individual rats of the different diet groups. In contrast, in many expression-profiling studies RNA samples are pooled, and group average expression profiles are presented, without knowledge on individual variation. The set of genes selected in our study shows statistical significance, indicating that the use of duplo hybridization experiments of six or seven rats per group is appropriate to identify unambiguously *in vivo* diet-induced differential expression. It appears that expression changes <2-fold were mostly not statistically significant. In these cases the diet-induced variation is too small and cannot be distinguished from biological variation, which is inevitable with *in vivo* gene expression profiling.

Two different approaches were used to select differentially expressed genes. The first method consisted of a straightforward pair-wise comparison of the different diet groups, where genes were selected that showed a 2-fold or larger differential expression. These expression changes were all statistically significant different in a Student's *t*-test. PCA was used as an alternative for the pair-wise comparisons. The PCA analysis has the clear advantage that it allows for direct comparison of all four diet groups. It identifies changes that contribute most to the differences between the groups, but it is difficult to set a cut-off parameter for gene selection. Our data set provides insight in this matter. By using a selection of 2% of most contributing genes either on the PC1 axis, or in the first three dimensions, exactly the same genes were identified as by using the bi-plots with the exception of ion transport regulator *Fxyd4*. This may be due to the fact that the *Fxyd4* gene is not regulated at all in the calcium versus control diets.

All genes that are differentially expressed in response to heme and/or calcium diets were reported previously to display expression in the intestine. For three genes, being *Mptx*, calcium activated chloride channel 3 (*Clca3*) and intelectin (*Itln*), more than one cDNA clone (respectively, 14, 6 and 3 cDNAs) was present on our array. For all three genes we have confirmed that the redundant clones show highly similar diet-modulated effects.

Evaluation of the biological function of the different genes learns that some expression changes do not give a clear mechanistic explanation for the observed physiological effects, i.e. irritation and hyperproliferation of colonic epithelium. For example, the significance of the effects on genes involved in digestion, intestinal fatty acid binding protein (*FABP2*) and carboxyl ester lipase (*CEL*) are unclear. Interestingly, *FABP2* was identified as the major responsive gene in a recent nutrigenomics study assessing the effects of dietary zinc (12). The gene expression changes that are observed for *Clca3*, a truncated member of the calcium activated chloride channel family, and that of mouse *Itln*, reported to be specifically expressed in intestinal paneth cells (13), are also difficult to explain, since the function of these proteins is unknown.

The genetic effects on the homolog of mouse DnaJ, subfamily C, member 7 (*Dnajc7*) are suggestive for changes in

cell cycle events. The alternative name for *Dnajc7* is tetratricopeptide repeat-containing gene 2 (*TRP2*), which refers to the TRP repeats of this protein. *TRP2*-containing proteins are identified in various organisms, including bacteria, yeast and animals and are known to be involved in cell-cycle control (14). However, its function is unclear and hence the significance of heme- and calcium-induced regulation awaits further studies.

A number of identified genes are clearly linked with alterations of cell turnover processes of colonic epithelium. The gene carbonic anhydrase I (*CA1*), specifically expressed in colonic epithelium, is associated with epithelial proliferation and cell death (15). A rat study on colonic epithelial cell turnover and expression of *CA1* in experimental colitis, induced by dietary supplementation of dextran sulfate sodium, has shown that during active colitis *CA1* levels are decreased, while in the regenerative phase involving increased proliferation and differentiation of the epithelium, up-regulation of *CA1* was observed (16). Similarly, a decrease in *CA1* expression has been shown in active human ulcerative colitis, while humans with colitis in remission show restored *CA1* expression levels (17). In line with these observations, up-regulation of *CA1* in the heme and heme/calcium-fed rats indicates that increased proliferation takes place in the colonic epithelium, possibly as part of the regenerative processes. The observed up-regulation of cytokeratin 21 (*Krt21*) in response to heme provides another indication for the heme-induced modulation of cell turnover processes. *Krt21* is a prominent component of rat intestinal epithelium and is the rat homolog of human keratin 20 (18). *Krt20* is used as a histodiagnostic marker for detection and staging of colorectal cancer (19). Elevation of *Krt21*, as observed in our study, reflects alterations in proliferation and differentiation of colonic epithelium.

The FXYD domain-containing ion transport regulator 4 is a member of a family of single transmembrane small ion transport regulators ($Na^{+}K^{+}$-ATPase). Although a role in cell turnover is not known for *Fxyd4*, it is suggestive that other members of the family are associated with tumors. *Fxyd3* (*Mat8*) is a breast tumor marker (20) and *Fxyd5* was shown to down-regulate E-cadherin and promote metastasis (21). Rat *DMBT1*, previously CRP-ductin, has also been implicated in epithelial differentiation and a role in the carcinogenesis of epithelial tumors has been proposed (22,23). Heme-induced modulation of *DMBT1* provides additional evidence that heme affects epithelial cell turnover, inhibited by calcium.

It should be noted that we also printed several classical markers for cell proliferation on our array, including cyclin D1 (*ccnd1*), ornithine decarboxylase (*odc*), cell division cycle 20 (*cdc20*) and proliferating cell nuclear antigen (*pcna*). These show only minor responses to heme and calcium and average signal intensities just above background. Such low signals, which suggest low expression of these genes in colonic mucosa, are difficult to interpret, since expression differences are close to the noise. Nevertheless, they tend to have increased expression in response to heme, while expression remains unaffected in calcium-fed animals. Average fold changes in response to, respectively, heme and calcium were as follows: *ccnd1* +1.3, −1.1; *odc* +1.5, +1.1; *cdc20* +1.4, −1.1; *pcna* +1.5, 1.0.

The strongest genetic effect induced by heme or calcium was observed for *Mptx*. We have identified previously *Mptx* as a novel rat gene, belonging to the family of pentraxins (6). This family consists of highly conserved pentameric proteins with high sequence homology (24). Two other short (25 kDa) pentraxins are known: *SAP* and *CRP*. Several distinct larger pentraxins have been identified, containing a C-terminal pentraxin-domain fused to an unrelated N-terminal domain, which are called 'long' pentraxins (>45 kDa) (25). SAP and CRP are both serum proteins that are produced mainly by hepatocytes. The function of these pentraxins is still under investigation, but there is growing evidence that they play a role in recognition and clearance of pathogenic targets, and auto-antigens from dead host cells (26,27). They display calcium-dependent ligand binding to these targets and mediate their elimination by recruiting the complement system. Clearance of auto-antigens from necrotic or apoptotic cells contributes to restoration of normal structure and function of injured tissues. In this report we show that *MPtx* is predominantly expressed in colonic mucosa. Its strong regulation by heme and calcium suggests that its function is associated to cell turnover processes, and like SAP and CRP, it might be involved in binding and clearance of mucosal epithelial cell debris.

Recently, a new short pentraxin has been identified in frogs by Peavy *et al*. (28), which was exclusively expressed in the oviduct. It is named jeltraxin and was isolated from frog egg jelly. It was speculated that its function is related to maintaining the structure of the jelly. Therefore, we investigated other mucosal epithelial tissues, such as lung and kidney, for *Mptx* expression. However, the results show predominant expression of *Mptx* in colonic epithelium. As such, it is unlikely that Mptx has a general mucosa-stabilizing function, but rather is associated to colon specific cell turnover.

The observed genetic changes in colonic mucosa strengthen our hypothesis that dietary heme damages colonic epithelial cells, which leads to a compensatory increased proliferation, a risk factor for tumorigenesis. Clearly, the genetic effects of calcium are opposite, and moreover, calcium inhibits the heme-induced physiological and genetic effects. Remarkably, Mptx expression is high in normal colon and strongly reduced upon epithelial damage. In contrast, the related pentraxins SAP and CRP are strongly up-regulated in response to stress induced by pathogenic targets. This contrast suggests that Mptx distinguishes from the other two pentraxins, not only in its site of expression, but possibly also in its biological function. We speculate that this might be related to the fact that Mptx may exhibit its biological function in a restricted area of the body, namely colonic tissue, while SAP and CRP act systemically. In colonic tissue cell death and regeneration are healthy, continual processes, while cell death induced by pathogenic targets in serum requires triggering of immune responses of the host.

Taken together, our *in vivo* nutrigenomics study shows that dietary heme and calcium modulate the gene expression profile of several genetic markers for epithelial proliferation and differentiation, indicative for altered mucosal cell turnover. *Mptx* appears to be the most sensitive gene to the diets, exhibiting 30-fold differential expression in colonic epithelium. The molecular basis for this unusual strong response is at present unclear, but seems to be specifically related to colonic mucosa. We propose *Mptx* as a new colon-specific marker for mucosal cell integrity.

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