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Gene expression in human small intestinal mucosa in vivo is mediated by iron-induced oxidative stress

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Troost, Freddy J., Robert-Jan M. Brummer, Guido R. M. M. Haenen, Aalt Bast, Rachel I. van Haaften, Chris T. Evelo, and Wim H. M. Saris. Gene expression in human small intestinal mucosa in vivo is mediated by iron-induced oxidative stress. *Physiol Genomics* 25: 242–249, 2006. First published February 7, 2006; doi:10.1152/physiolgenomics.00114.2005.—Iron-induced oxidative stress in the small intestine may alter gene expression in the intestinal mucosa. The present study aimed to determine which genes are mediated by an iron-induced oxidative challenge in the human small intestine. Eight healthy volunteers [22 yr(SD2)] were tested on two separate occasions in a randomized crossover design. After duodenal tissue sampling by gastroduodenoscopy, a perfusion catheter was inserted orogastrically to perfuse a 40-cm segment of the proximal small intestine with saline and, subsequently, with either 80 or 400 mg of iron as ferrous gluconate. After the intestinal perfusion, a second duodenal tissue sample was obtained. Thiobarbituric acid-reactive substances, an indicator of lipid peroxidation, in intestinal fluid samples increased significantly and dose dependently at 30 min after the start of perfusion with 80 or 400 mg of iron, respectively ($P < 0.001$). During the perfusion with 400 mg of iron, the increase in thiobarbituric acid-reactive substances was accompanied by a significant, momentary rise in trolox equivalent antioxidant capacity, an indicator of total antioxidant capacity ($P < 0.05$). The expression of 89 gene reporters was significantly altered by both iron interventions. Functional mapping showed that both iron dosages mediated six distinct processes. Three of those processes involved G-protein receptor coupled pathways. The other processes were associated with cell cycle, complement activation, and calcium channels. Iron administration in the small intestine induced dose-dependent lipid peroxidation and a momentary antioxidant response in the lumen, mediated the expression of at least 89 individual gene reporters, and affected at least six biological processes.

transcriptomics; intestine; epithelium; microarrays

IT IS GENERALLY ACCEPTED THAT oxidative stress in the intestine is an etiological factor in the pathophysiology of several inflammation-associated disorders, such as inflammatory bowel disease and celiac disease (11, 15–17, 28). Diet-related components may be important mediators of oxidative damage. Previous studies (9, 21) showed that iron supplements induce oxidative damage to the small intestine, owing to its catalyzing role in Fenton chemistry, which results in the production of highly reactive hydroxyl radicals. Furthermore, iron intake was

associated with impairment of the barrier function of the small intestine epithelium (14), the development of inflammation in ulcerative colitis (1, 19), a decrease in cell turnover, shortening of microvillus height and erosions of the microvilli in the duodenum (22), and the development of colon cancer (20, 26). Our laboratory showed previously that a single iron dose administered directly into the small intestine induces lipid peroxidation and the release of, yet unidentified, antioxidants in the lumen of the small intestine (24). The cellular mechanisms involved in the intestinal oxidative stress and defence responses are not clear. The rapidly evolving microarray technology provides a sophisticated method to synchronously monitor the expression of thousands of genes to obtain information about the regulation of numerous biochemical processes in the intestinal mucosa on the transcriptional level. This technique has the potential to elucidate complex biochemical or physiological pathways, such as intestinal oxidative stress and its consequences. The combination of the microarray technology with novel, invasive human intervention models, applied in the present study, provides a unique and valuable tool to investigate nutrient-gene interactions in vivo in the human intestine.

The present study aimed to assess iron-induced oxidative damage and the antioxidant response in the human small intestine in vivo after administration of commonly used iron substitution dosages and to determine the effects of this intestinal oxidative stress on transcriptome profiles in the small intestine.

MATERIALS AND METHODS

Subjects

Eight healthy nonsmoking volunteers [22 yr(SD2), 7 men, 1 woman] without a history of gastrointestinal disorders participated in the study. Volunteers were free of medication and in good physical health. All subjects gave their written, informed consent before participation. The study protocol was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Protocol

This study was designed to conform to a randomized crossover design. Each subject was tested on 2 separate days. On each test day, tissue samples from the duodenum were obtained before and after small intestinal perfusion experiments. On each day, one iron dosage was administered directly into the duodenum through a perfusion catheter, as described below. In the intestinal perfusion experiments, intestinal fluid samples were obtained at 15-min intervals during saline perfusion as baseline samples and during the subsequent iron perfusion.

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After an overnight fast, baseline duodenal biopsies, taken by standard forceps in the proximal horizontal part of the duodenum (15 cm distally from the pylorus), were obtained by gastroduodenoscopy. All gastroduodenoscopies were performed without giving any kind of sedatives to the subjects. Biopsies were imbedded in tissue-tek (Electron Microscopy Sciences, Washington, PA), snap-frozen in melting isopentane and liquid nitrogen, respectively, and stored at -80°C until analysis. After obtaining the mucosal samples, a guide wire was inserted with the distal tip positioned about 60 cm distally from the pylorus, facilitated by the duodenoscopy. After retraction of the endoscope, a double lumen perfusion catheter (home made from PVC tubing, inner diameter 2.5 mm, outer diameter 4 mm) was inserted over the guide wire into the proximal small intestine with the tip of one lumen, which served as an injection port, located in the proximal descending duodenum and the tip of the second lumen, with three small holes in the last 3 cm to serve as a sample port, located 40 cm distally from the injection port. After being positioned, the guide wire was removed. The whole procedure was performed under short-interval fluoroscopic control. To check positioning of the injection port in the duodenum throughout the experiment, intraluminal pH was continuously measured at 5 cm proximal of the injection port by a pH electrode (Single Crystal Antimony Multi-Use pH Catheter, Syntectics Medical, Queluz, Portugal). The 40-cm segment of the proximal small intestine was perfused with saline at 10 ml/min using a peristaltic pump for 195 min to obtain steady-state conditions. After reaching steady state, either 80 or 400 mg of iron as ferrous gluconate dissolved in 300 ml saline was perfused. The iron solutions were perfused over a 30-min period. Finally, saline was perfused for another 60 min. All in-going solutions were kept on 37°C in a water bath. Throughout the perfusion experiment, intestinal fluid samples were obtained continuously using the lever properties of the catheter. After the initial 180-min adaptation period, intestinal fluid was levered through the sample lumen into a beaker placed in melting ice. Intestinal fluids were pooled over 15-min intervals and stored at -80°C until analysis.

Fifteen minutes after the perfusion experiment, the perfusion catheter was removed and a second gastroduodenoscopy was performed to obtain tissue samples from the same intestinal region after the iron challenge. These tissue samples were obtained 3–4 cm distally from those taken in the morning. The exact position of the tissue sampling in the morning was achieved by visual inspection of the gut mucosa. A red color from the mucosa, different from the surrounding tissue, showed the exact position of earlier tissue sampling. The entire protocol was repeated on another day, ~ 2 wk after the first experiment, to investigate the effects of the other iron dosage. The administration of both dosages was in random order.

Gene Expression Analysis

Gene expression profiles were determined in all tissue samples. Histological staining was done in a subset of the samples to check the composition of the samples. The mucosal tissue samples contained epithelium from several intestinal villi and also lamina propria. Total RNA was isolated from each tissue sample, and RNA quality was assessed by SDS-PAGE gel electrophoresis. After quality check, only tissue samples from five subjects provided good-quality RNA. The tissue samples from three subjects were excluded from the microarray analyses. For budget reasons, the RNA samples from the remaining five subjects, all men, were randomly allocated to one of two pools. Samples from two subjects were pooled in *pool 1*, and samples from three subjects were pooled in *pool 2*. On each pooled sample, a microarray analysis to determine gene expression profiles was carried out. Samples were obtained before and immediately after the intestinal perfusion with a low or a high iron dosage, as described above.

cRNA Preparation and In Vitro Transcription

Ten micrograms of total RNA was used as starting material for the cDNA preparation. The first- and second-strand cDNA synthesis was

performed using the SuperScript II System (Invitrogen) according to the manufacturers instructions except using an oligo-dT primer containing a T7 RNA polymerase promoter site. Labeled cRNA was prepared using the BioArray high-yield RNA transcript labeling kit (Enzo, Farmingdale, NY). Biotin-labeled CTP and UTP (Enzo) were used in the reaction together with unlabeled NTPs. After the in vitro transcription (IVT) reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array Hybridization and Scanning

Fifteen micrograms of cRNA was fragmented at 94°C for 35 min in a fragmentation buffer containing (in mM) 40 Tris-acetate (pH 8.1), 100 KOAc, and 30 MgOAc. Before hybridization, the fragmented cRNA in a $\times 6$ SSPE-T hybridization buffer [1 M NaCl, 10 mM Tris (pH 7.6), 0.005% Triton] was heated to 94°C for 5 min and subsequently to 45°C for 5 min before loading onto the Affymetrix HG U133A probe array cartridge. The probe array was then incubated for 16 h at 45°C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station. The probe array was exposed to 10 washes in $\times 6$ SSPE-T at 25°C followed by 4 washes in $\times 0.5$ SSPE-T at 50°C . The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, final concentration 2 mg/ml (Molecular Probes, Eugene, OR) in $\times 6$ SSPE-T for 30 min at 25°C , followed by 10 washes in $\times 6$ SSPE-T at 25°C . Subsequently, an antibody amplification step was performed, using normal goat IgG as blocking reagent, final concentration 0.1 mg/ml (Sigma, Copenhagen, Denmark), and biotinylated anti-streptavidin antibody (goat) to obtain a final concentration of 3 mg/ml (Vector Laboratories, Burlingame, CA). This was followed by a staining step with a streptavidin-phycoerythrin conjugate, final concentration 2 mg/ml (Molecular Probes, Eugene, OR), in $\times 6$ SSPE-T for 30 min at 25°C , and 10 washes in $\times 6$ SSPE-T at 25°C . The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (Hewlett Packard GeneArray scanner G2500A). The readings from the quantitative scanning were analyzed with the Affymetrix gene expression analysis software. All microarray data are publicly available on the ArrayExpress website, as described in RESULTS.

Each gene reporter on the U133A chip was represented by 11 pairs of 25-mer oligodeoxyribonucleotide probes. Each probe pair consists of a perfect-match oligonucleotide that precisely matches the gene sequence and a mismatch oligonucleotide that differs only in one single nucleotide mismatch in the center position. The data from the different sets of probe pairs were used to perform the statistical analyses. For comparison from array to array, all signal data from the quantitative readings were scaled to a global intensity of 150, as previously published (4, 23).

The signal intensities from the microarrays were scaled and further analyzed using the Affymetrix Microarray Suite version 5.0 software (Affymetrix, Santa Clara, CA). Relative mRNA changes between microarrays were expressed as differences or delta compared with baseline samples for the 80- and 400-mg iron challenge, respectively. The definition of increase, decrease, or no change of expression for individual gene reporters was based on P values of the comparisons of the normalized signal intensities, with $P < 0.05$ as cutoff for change calls. Gene reporters that were either up- or downregulated in the same direction in both pools were further investigated. If the expression of a gene reporter was increased in one pool while it did not change or decrease in the other pool, it was assumed that the gene expression was not mediated by the iron interventions. Likewise, only those gene reporters that were mediated in the same direction by both the low- and the high-iron dosage interventions were further investigated.

To allow functional mapping of expression profiles, the annotation of individual probes with the Swissprot [<http://us.expasy.org/sprot/sprot-top.html> (5)] database identification (ID) for the corresponding

protein was used. Using these IDs, expression changes for each of the four pooled samples were studied using functional MicroArray pathway profiles (MAPPs) available from the GenMapp initiative (<http://www.genmapp.org/>) with GenMapp version 1.0 beta software (7). MAPPs generated from the Gene Ontology database [<http://www.geneontology.org/>] (6), the G-Protein Coupled Receptor Database (<http://www.gpcr.org/>), plus a small number of MAPPs based on the KEGG database (<http://www.genome.ad.jp/kegg/>) and MAPPs specifically designed for GenMapp (so called local MAPPs) were used. MappFinder software was used to select the MAPPs with relatively high numbers of differentially expressed gene reporters with a fold change of at least 2.3, as was indicated by the individual Z-scores. The Z-score increases when relatively higher numbers of changing gene reporters relative to the number of genes present on the MAPP that are represented on the array are found on MAPPs. MAPPs were selected for further study when all four samples reached an arbitrary Z-score of at least 1 on that MAPP.

Real-time PCR. To validate the observed mRNA changes from the microarray analyses, real-time PCR was performed on seven genes. cDNA was synthesized from single samples previously analyzed on GeneChips. The microarray gene reporter expression profiles of the seven genes, with Unigene cluster IDs nm_005410, nm_001924, nm_002306, nm_005118, nm_00075, nm_003355, and nm_003056, were compared with real-time PCR. For this purpose, real-time PCR measurements were done in the pooled samples that were also used for the microarray experiments. Additionally, real-time PCR analyses were carried out on all individual samples. Reverse transcription was performed using Superscript II RT (Invitrogen). 1 µg of total RNA and 3 µl of 150 pmol/µl random nonamer-primer in a total volume of 12 µl was incubated 4 min at 70°C and chilled on ice. After adding 4 µl of first-strand buffer (from supplier), 2 µl of DTT (0.1 M), 1 µl of dNTP mix (10 mM), and 1 µl of SuperScript RT II (200 U/µl), the reaction was incubated 1 h at 45°C and finally for 15 min at 70°C. The cDNA was diluted 1:20 for use in real-time PCR. Real-time PCR analysis was performed on selected genes using primers, designed with PrimerExpress software from Applied Biosystems. Triple determinations were performed on ABI PRISM 7000 sequence detection system using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR reaction consisted of 12.5 µl of SYBR Green PCR Master Mix, 300 nM of forward and reverse primers, and 2.0 µl of 1:20 diluted template cDNA in a total volume of 25 µl. The reaction was thermocycled using the default settings of "ABI Prism 7000 SDS Software 1.0": 2 min at 50°C, 10 min at 95°C, followed by 40 rounds of 15 s 95°C and 1 min at 60°C. A dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 65 to 95°C. All samples were normalized to GAPDH, as was applied previously (4).

Biochemical Analysis of Intestinal Fluid Samples

Thiobarbituric acid-reactive substances assay. The determination of thiobarbituric acid-reactive substances (TBARS) was based on the formation of a coloured adduct of malondialdehyde with 2-thiobarbituric acid. A 100-µl sample was added to 900 µl of a reagent [containing 0.12 M 2-thiobarbituric acid, 0.32 M *O*-phosphoric acid, 0.68 mM butylated hydroxytoluene, and 0.01% (mass/vol) EDTA]. The mixture was incubated for 1 h at 100°C in a water bath. After cooling, the malondialdehyde products were extracted with 500 µl of butanol. Thirty microliters of the butanol layer was injected on a HPLC system (Agilent, Palo Alto, CA), equipped with a fluorescence detector, set on excitation wavelength of 532 nm and emission wavelength of 553 nm, and a Nucleosil C18 column, 150 × 3.2 mm, particle size 5 µm (Supelco). Samples were eluted with 65% (vol/vol) 25 mM phosphate buffer pH 4.8, 35% (vol/vol) methanol. A calibration curve was constructed using malonaldehyde bis(diethylacetal) as standard.

Antioxidant capacity assay. The antioxidant capacity assay was carried out as described previously (3) with some modifications. The 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) radical was produced by incubating a solution of 0.23 mM ABTS and 2.3 mM 2,2'-azobis(2-amidinopropane)dihydrochloride (ABAP) in 100 mM sodium phosphate buffer (pH 7.4) at 70°C until the absorption of the solution at 734 nm was between 0.680 and 0.720. Deproteination was obtained by mixing the sample with an equal volume of a solution of 10% (wt/vol) trichloro-acetic acid. In the reaction of antioxidants with the blue/green ABTS radical, the blue-green color disappears. This decolorization after 5 min was determined spectrophotometrically at 734 nm. The reduction in absorbance is related to that of trolox, a synthetic, hydrophilic vitamin E analog, which gives the trolox-equivalent antioxidant capacity (TEAC) value. The TEAC value was calculated as molar trolox equivalents of the sample.

Statistics

Statistical analyses of the scaled microarray data were performed with the Affymetrix Microarray Suite version 5.0 software package, applying Wilcoxon's signed rank tests to detect differences between arrays. Time effects of malondialdehyde and TEAC within each iron intervention, compared with baseline values obtained at time = 0, were investigated with repeated-measures ANOVA. Significant time differences were located with Scheffé's post hoc analyses, including Bonferroni correction for multiple comparisons. Univariate ANOVAs were used to assess differences between the two iron dosages. Differences were regarded as significant if $P < 0.05$.

RESULTS

Gene Expression

All array data are publicly available on the ArrayExpress website (<http://www.ebi.ac.uk/arrayexpress/>) and can be accessed by logging in to the system with username "e-mexp-325" and password "6iud2ebi." After normalization of the microarray signals, 8,542 gene reporters showed a significant expression in both pools at baseline. Another 476 gene reporters were marginally expressed according to the Affymetrix default cutoff values, whereas the remaining 13,271 reporters were not expressed in the duodenal tissue samples.

The microarray analyses showed that the expressions of 89 gene reporters were consistently increased or decreased compared with baseline by both iron challenges in both pools; 28 gene reporters were increased (Table 1), whereas 61 gene reporters were decreased (Table 2).

Mapping the individual gene reporters to shared biological pathways using MAPPFinder software revealed that, considering the biological processes observed in both pools, six biological processes were mediated by both iron dosages. Three of those six processes involved G-protein-coupled receptors (GPCRs), whereas the other three processes were associated with complement activation, calcium channels, and cell cycle. The processes, which were mediated by the two interventions in both sample pools, are listed in Table 3. Table 3 shows the smallest number of gene reporters mediated by any pool in any intervention. Furthermore, it shows the number of gene reporters involved in each process, which were represented on the microarray chip, and the total number of genes involved in each process, which was defined by the databases described in METHODS.

Real-time PCR analyses of seven randomly selected genes showed good similarity with microarray analyses of the same genes, considering the direction of changes in gene reporter

Table 1. *Gene reporters consistently downregulated by perfusion of a small intestinal segment with 80 and 400 mg of iron on 2 separate test days*

Probe ID	Gene Title
200617_at	KIAA0152 gene product
201009_s_at	Thioredoxin-interacting protein
201275_at	Farnesyl diphosphate synthase
201790_s_at	7-dehydrocholesterol reductase
201876_at	Paraoxonase 2
202017_at	Epoxide hydrolase 1, microsomal (xenobiotic)
202025_x_at	Acetyl-Coenzyme A acyltransferase 1
202122_s_at	Mannose 6 phosphate receptor binding protein
202433_at	UDP-galactose transporter related
202793_at	
203414_at	Monocyte to macrophage differentiation-associated
203633_at	Carnitine palmitoyltransferase 1, liver
203943_at	Kinesin family member 3B
204039_at	CCAAT/enhancer binding protein (C/EBP), alpha
204233_s_at	Choline kinase
204266_s_at	Choline kinase
204429_s_at	Solute carrier family 2 (facilitated glucose/fructose transporter), 5
204544_at	Alpha integrin binding protein 63
204607_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
204760_s_at	Nuclear receptor subfamily 1, group D, member 1
204997_at	Glycerol-3-phosphate dehydrogenase 1 (soluble)
205073_at	Cytochrome P450 subfamily III(arachidonic acid epoxigenase) pp 2
205749_at	Cytochrome P450, subfamily I(aromatic compound-inducible), pp 1
208308_s_at	Glucose phosphate isomerase
208684_at	Coatomer protein complex, subunit alpha
208998_at	Uncoupling protein 2 (mitochondrial, proton carrier)
209122_at	Adipose differentiation-related protein
209123_at	Quinoid dihydropteridine reductase
209355_s_at	Phosphatidic acid phosphatase type 2B
209775_x_at	Solute carrier family 19 (folate transporter), member 1
209776_s_at	Solute carrier family 19 (folate transporter), member 1
209777_s_at	Solute carrier family 19 (folate transporter), 1
209782_s_at	D site of albumin promoter binding protein
210095_s_at	Insulin-like growth factor binding protein 3
210830_s_at	
211576_s_at	
211618_s_at	
211699_x_at	Hemoglobin, alpha 1
211745_x_at	Hemoglobin, alpha 2
211922_s_at	
213706_at	
213787_s_at	
214274_s_at	
216474_x_at	
216894_x_at	Cyclin-dependent kinase inhib. 1C (p57, Kip2)
217414_x_at	Hemoglobin, alpha 2
217478_s_at	
218094_s_at	Chromosome 20 open reading frame 35
218421_at	Hypothetical protein FLJ23239
218688_at	DKFZP586B1621 protein
219398_at	Protein FLJ20871 similar to FSP27
219534_x_at	Cyclin-dependent kinase inhib. 1C (p57, Kip2)
220070_at	Hypothetical protein FLJ13798
221529_s_at	Plasmalemma vesicle associated protein
221552_at	Lipase protein
221679_s_at	Lipase protein
221951_at	
31637_s_at	Nuclear receptor subfamily 1, group D, 1
38158_at	Extra spindle poles, <i>S. cerevisiae</i> , homolog of
45288_at	Lipase protein
65630_at	Similar to RIKEN cDNA 5530601119 gene,
209777_s_at	Solute carrier family 19 (folate transporter), 1

Probe identification numbers (Probe IDs) refer to the Affymetrix reference number of the measured probe pairs.

Table 2. *Gene reporters consistently upregulated by perfusion of a small intestinal segment with 80 and 400 mg of iron on 2 separate test days*

Probe ID	Gene Title
201362_at	NS1-binding protein
201368_at	
201427_s_at	Selenoprotein P, plasma, 1
201695_s_at	Nucleoside phosphorylase
201925_s_at	Decay accelerating factor for complement
203287_at	Ladinin 1
203574_at	Nuclear factor, interleukin 3 regulated
203725_at	Growth arrest and DNA-damage-inducible, alpha
203824_at	Transmembrane 4 superfamily member 3
203914_x_at	Hydroxyprostaglandin dehydro-genase 15-(NAD)
205769_at	Fatty-acid-Coenzyme A ligase, very long-chain 1
205771_s_at	A kinase (PRKA) anchor protein 7
205860_x_at	Folate hydrolase (prostate-specific membrane antigen) 1
205969_at	Arylacetamide deacetylase (esterase)
208921_s_at	Sorcin
208949_s_at	Galectin 3
209301_at	Carbonic anhydrase II
210177_at	Tripartite motif-containing 15
211548_s_at	Hydroxyprostagland dehydrogenase 15NAD
211962_s_at	
212130_x_at	
213372_at	
215363_x_at	
217761_at	SIPL protein
219508_at	Glucosaminyl (N-acetyl) transferase 3
220639_at	Hypothetical protein FLJ22800
222303_at	
40016_g_at	KIAA0303 protein

profiles following the iron interventions (Table 4). The real-time PCR measurements on individual samples showed that the results from the pooled samples represented those of most individual samples (Table 5). For six of the selected genes, all individual results showed a unidirectional response to the iron challenges, which was in agreement with the results from the pooled samples. The real-time PCR data from one volunteer did not show the same direction of response to the iron challenges (gene NM_003355, *pool 1, subject 1*; Table 5) compared with the pooled samples.

Table 3. *Processes in small intestinal mucosa affected by iron administration*

Process	MAPP	Gene Reporters Changed/Measured/Total
Cell cycle	KEGG	6/9/104
GPCRs, class C	GPCRDB Mapp	3/12/18
Calcium channels	Local MAPPs	7/22/28
Complement activation, classical pathway	Local MAPPs	4/12/16
Peptide GPCRs	Local MAPPs	9/45/69
GPCRs, class A	GPCRDB MAPP	22/112/289

Processes most strongly mediated by the 2 iron interventions in both pools are identified by Mappfinder, as described in MATERIALS AND METHODS. The *middle* column shows which database was used for the identification of the biological pathway. The number of mediated gene reporters that changed more than 2.3-fold by both iron interventions in both pools, the total number of genes involved in each process and represented on the microarray chips (HG U133A), and the total number of genes involved in each process as specified by the consecutive databases are listed in the *right* column. GPCR, G-protein-coupled receptor; MAPP, MicroArray pathway profiles.

Table 4. Comparison of RT-PCR and microarray results

GenBank Accession No.		1 L	1 H	2 L	2 H
nm_005410	RT-PCR	1.30	1.90	2.04	1.56
	Microarray	1.28	1.68	1.39	1.24
nm_001924	RT-PCR	1.68	2.92	2.56	2.44
	Microarray	1.62	1.76	1.69	1.98
nm_002306	RT-PCR	1.56	1.96	2.26	3.20
	Microarray	1.46	1.66	1.32	1.39
nm_005118	RT-PCR	7.06	13.53	8.32	11.80
	Microarray	2.12	2.05	2.29	1.95
nm_000775	RT-PCR	-1.31	-1.29	-1.21	-1.36
	Microarray	-1.38	-1.37	-1.12	-1.35
nm_003355	RT-PCR	-1.29	-1.17	-1.41	-1.44
	Microarray	-1.28	-1.18	-1.36	-1.62
nm_003056	RT-PCR	-1.76	-1.75	-1.57	-1.62
	Microarray	-1.71	-1.76	-1.44	-1.77

RT-PCR analysis of selected genes and microarray data are from the same genes. Data are expressed as fold changes compared with baseline. The first column (1 L) shows the gene expression data from *pool 1* after administration of the low dosage of iron compared with baseline; column 1 H shows data from *pool 1* after administration of the high iron dosage. 2 L and 2 H columns show the data from *pool 2* after administration of the low and high iron dosage, respectively.

TBARS and TEAC

To check steady-state conditions, TBARS and TEAC were measured at 30 and 15 min and immediately before iron perfusion was started. TBARS concentrations were 0.06, 0.06, and 0.20 μM , and TEAC values were 577, 520, and 597 at 30, 15, and 0 min before starting the 80-mg iron perfusion, respectively. Before the 400-mg iron perfusions were started, the TBARS concentrations were 0.33, 0.29, and 0.40 μM and the TEAC values were 822, 819, and 806 μM , respectively, at 30, 15, and 0 min before iron perfusion, showing that TBARS and TEAC values were, indeed, in a steady state.

The TBARS concentration in intestinal fluid samples after administration of 80 mg of iron increased significantly from 0.2 μM (SD0.16) at baseline to 2.9(SD1.2), 2.3(SD1.5), and 2.4 μM (SD1.4) at 30, 45, and 60 min, respectively, after the start of the iron perfusion ($P < 0.0001$). The TBARS concentration after administration of 400 mg of iron increased significantly from 0.4 μM (SD0.5) at baseline to 3.8(SD1.1),

Fig. 1. Thiobarbituric acid-reactive substances (TBARS) concentrations, an indicator of lipid peroxidation, in intestinal perfusion samples collected over 15-min intervals after perfusion of a 40-cm segment in the proximal small intestine in 8 subjects. Baseline values at time = 0 were obtained after 180 min of saline perfusion. Saline containing 80 (diamonds) or 400 mg (squares) of elemental iron as ferrous gluconate was perfused for 30 min, followed by saline perfusion for 60 min at 10 ml/min. Values are means $\pm$ SE. *Significant differences compared with baseline ($P < 0.001$).

4.1(SD1.4), and 3.8 μM (SD1.5) at 30, 45, and 60 min after the start of the iron perfusion ($P < 0.001$). Univariate ANOVA showed that TBARS production after administration of 400 mg of iron was significantly higher compared with 80 mg of iron ($P < 0.001$; Fig. 1).

The TEAC concentration after administration of 80 mg of iron increased from 597 μM (SD187) at baseline to 1,032 μM (SD609) at 30 min after the start of the iron perfusion. Statistical analysis did not detect an overall time effect. The TEAC concentration after administration of 400-mg iron intervention increased significantly from 806 μM (SD293) at baseline to 1,889(SD635) and 1,490 μM (SD413) at 30 and 45 min, respectively, after the start of the iron perfusion ($P < 0.05$).

Univariate ANOVA showed that TEAC after administration of 400 mg of iron was significantly higher than after 80 mg of iron ($P < 0.001$; Fig. 2).

DISCUSSION

In the present study, a recently developed intestinal perfusion protocol was combined with microarray technology to study nutrient-gut and nutrient-gene interactions in the human

Table 5. Comparison of pooled and individual RT-PCR results

	NM_005410	NM_001924	NM_000775	NM_002306	NM_003056	NM_005118	NM_003355
L <i>pool 1</i>	1.30	1.68	0.69	1.56	0.24	7.06	0.71
L <i>subject 1</i>	1.49	2.52	0.93	1.39	0.62	13.79	1.85
L <i>subject 2</i>	0.95	1.14	0.72	1.70	0.16	6.96	0.57
H <i>pool 1</i>	1.90	2.92	0.71	1.96	0.25	13.53	0.83
H <i>subject 1</i>	2.39	1.85	1.02	0.73	0.30	10.73	1.97
H <i>subject 2</i>	2.04	3.55	0.82	3.03	0.28	21.71	0.67
L <i>pool 2</i>	2.04	2.56	0.79	2.26	0.43	8.32	0.59
L <i>subject 1</i>	4.66	3.58	1.55	2.02	0.90	13.93	1.85
L <i>subject 2</i>	0.94	3.09	0.65	2.87	0.79	1.65	1.03
L <i>subject 3</i>	2.30	1.52	0.67	1.21	0.15	6.23	0.55
H <i>pool 2</i>	1.56	2.44	0.64	3.20	0.38	11.80	0.56
H <i>subject 1</i>	2.08	1.87	0.53	1.19	0.25	15.43	0.63
H <i>subject 2</i>	1.28	2.29	0.93	3.72	0.35	5.71	0.86
H <i>subject 3</i>	1.03	1.31	0.69	1.31	0.19	8.22	0.39

RT-PCR analysis of selected genes on pooled (*pool 1* and *pool 2*, respectively) and individual samples from each subject. Data, which are normalized to GAPDH, are expressed as fold changes compared with baseline. L, changes in gene expression induced by administration of the low iron dosage; H, changes in gene expression induced by administration of the high iron dosage.

Fig. 2. Trolox-equivalent antioxidant capacity (TEAC) in intestinal perfusion samples collected over 15-min intervals after perfusion of a 40-cm segment in the proximal small intestine in 8 subjects. Baseline values at time = 0 were obtained after 180 min of saline perfusion. Saline containing 80 (diamonds) or 400 mg (squares) of elemental iron as ferrous gluconate was perfused for 30 min, followed by saline perfusion for 60 min at 10 ml/min. Values are means $\pm$ SE. *Significant differences compared with baseline ($P < 0.05$).

small intestine to synchronously investigate several intestinal responses to luminal oxidative challenges provoked by different iron dosages. Acute exposure of the proximal small intestine to either a low or a high dosage of ferrous iron induced dose-dependent lipid peroxidation in the intestinal lumen and a synchronous increase in luminal antioxidant capacity. These parameters were measured in intestinal fluid samples from all eight volunteers, in contrast to the microarray analyses, which were done on the samples from five volunteers. The mean TBARS and TEAC values from all eight volunteers did not change in only the five volunteers that were included in the microarray measurements (data not shown). The acute, single-dose, iron challenges mediated gene expression of a number of gene reporters. Both iron interventions strongly affected at least six distinct biological pathways on the transcriptional level.

We recently showed that perfusion of a 40-cm duodenum segment with 80 mg of iron as ferrous sulfate induced lipid peroxidation and the release of an unknown antioxidant. The rise in antioxidant capacity following iron ingestion was not caused by ex vivo TBARS formation during the analytical assay, cell lysis of epithelial cells, glutathione, uric acid, or vitamin C and was of nonprotein origin (24). The present study, applying ferrous gluconate, confirmed the catalyzing effects of ferrous iron administration in the small intestine to induce oxidative damage and the release of antioxidants into the intestinal lumen. At baseline, during the initial saline perfusion, TBARS were only marginally present. After ingestion of either the low or the high iron dosage, TBARS concentrations increased in a dose-dependent manner. This increase was accompanied by a similar dose-dependent increase in TEAC. In the view of the highly diluted intestinal fluid with the saline solution used for perfusion of the intestinal segment, the TBARS and TEAC concentrations were very high; all samples consisted mainly of the in-going saline solution mixed with small amounts of intestinal secretions. This indicates that the iron administration resulted in powerful antioxidant responses. In an attempt to identify the origin of the antioxidant response, NMR spectra from samples with high and low antioxidant capacity were taken. Although differences between spectra were observed, it was not possible to identify the

compound(s) responsible for these differences (data not shown).

The oligonucleotide microarray enabled one to simultaneously monitor the expression in vivo of more than 20,000 gene reporters. Among numerous processes that were slightly mediated by the iron interventions, six distinct processes were strongly regulated, as listed in Table 3. Three of these six processes involved GPCRs. However, two of these three processes were identified using the GPCRDB database, whereas one was identified by local MAPPS, as was described in METHODS. We cannot exclude that some gene reporters were attributed to one process in the GPCRDB database and at the same time to the GPCR process identified by local MAPPS, hence causing an overestimation of the genes involved in GPCR-associated processes. However, from the results, it is clear that GPCRs were strongly mediated by the iron interventions. GPCRs comprise the largest superfamily of proteins in the body. Ligands for these receptors are biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions, and proteases. All GPCRs are able to recruit and regulate the activity of intracellular G proteins, which regulate a large array of intracellular processes (13). The observation that GPCRs were regulated by iron-induced oxidative stress may indicate a yet unknown function of G-protein-associated processes in response to cellular stress. The observation that complement activation is regulated by the iron administrations suggests that the iron-induced oxidative damage triggered immune activation in the duodenal mucosa. Complement activation has previously been associated with mucosal damage in ulcerative colitis (25), whereas it was also established that mucosal damage owing to autologous complement activation can be prevented by endogenous regulatory mechanisms (2). Furthermore, it was shown that intestinal epithelial cells are able to produce components of complement activation and that this production can be mediated by cytokines in the intestinal epithelium (2). Increased activation of complement activation, as observed in the iron perfusion experiments, may induce complement-induced damage. Future studies will need to elucidate the consequences of complement activation in duodenal mucosa. The functional role of the cell cycle- and calcium channel-related processes, which were affected by the iron interventions, is not yet described to our knowledge and needs to be elucidated. Interestingly, it was shown that mice fed a low-selenium diet, which is a trace mineral with antioxidant properties, appeared to have an increased activation of genes involved in cell cycle control (18). This suggested that selenium mediates cell cycle control, which may account for its anti-tumorigenic properties. The present observation that cell cycle was mediated by iron-induced oxidative stress suggests that this process mediates the initiation of carcinogenesis if the oxidant/antioxidant balance is disturbed.

Genes involved in the regulation of iron absorption mechanisms have drawn the attention of many investigators, especially in the view of their causal role in the pathogenesis of hereditary hemochromatosis. Several proteins are involved in the direct regulation of iron uptake from the duodenal lumen. Divalent metal transporter 1 (DMT1), a transmembrane transporter for divalent metals, including ferrous iron, plays an important role in the absorption of iron from the gut lumen into enterocytes, whereas ferroportin1 is a transmembrane iron exporter molecule involved in the basolateral iron transport

from enterocytes into the circulation. In a recent study in mice (8), iron supplementation for 2 wk downregulated the levels of transcripts of DMT1, ferroportin1, and transferrin receptor 1, whereas feeding an iron-deficient diet resulted in an increase of these transcripts. Furthermore, duodenal gene expression of an iron-responsive element splice variant of DMT1 was significantly downregulated 1 h after intragastric iron administration in rats (10). In the present study, we showed that the gene expression of DMT1 and transferrin receptor 1 tended to decrease by ~25% in both sample pools following administration of the low and the high iron dosage into the duodenum, although these gene reporters were not regarded as significantly changed after statistical analysis. Unfortunately, ferroportin1 was not represented on the gene chip. Our findings contribute to the ongoing debate of the precise regulation of intestinal iron absorption. It is commonly acknowledged that high iron levels in the enterocytes downregulate DMT1 expression (10). The finding of the present study that acute iron ingestion may downregulate DMT1 and transferrin receptor 1 expression suggests that intestinal iron concentrations directly mediate transcription of these proteins, as was shown previously only for DMT1 expression (10, 29).

The genome-wide microarrays used yield information about the expression of a myriad of gene reporters. The major advantage of this technique is the ability to synchronously monitor many biological processes. However, the present study design has some drawbacks. The duodenal mucosal tissue samples comprised a variety of mucosal and submucosal cell types. Changes in gene expression that only occur in one cell type were, therefore, diluted by the presence of other cell types and, hence, may not have reached significance. In addition, gene expression levels differ between individuals. Pooling samples from different volunteers and the inclusion of only those gene reporters that were affected by both the low and the high iron dosage in both pools for further analysis, as was applied in the present investigation, reduced the effects of inter- and intra-individual variations in gene expression. We cannot exclude, however, that, using this approach, some relevant gene reporters that were mediated by the iron interventions were erroneously excluded from further analyses. On the other hand, the procedure followed to identify iron-mediated biological pathways ensured that the study results reveal the most important, strongest mediated processes.

Finally, the microarray assay itself could have induced false-positive signals due to cross-hybridization or nonspecific binding to the probe pairs. This seems highly unlikely in the view of previously published studies in which the data of Affymetrix microarray chips were successfully compared with real-time PCR analyses (12, 27). In the present study, we also confirmed the microarray data of seven genes that were mediated by the iron interventions with real-time PCR analyses. Table 4 shows that the changes in gene expression profiles in both pools after both iron interventions were roughly the same, either applying real-time PCR or microarray analyses. Some differences between the two methods were present, as can be observed from the results of gene nm_005118. Real-time PCR analysis showed a 13.53-fold change in the expression of this gene reporter, whereas microarray analysis showed only a 2.05-fold change in expression. This difference may be attributed to the low baseline level of this gene, which is a common concern in the interpretation of microarray results (12), espe-

cially if results are expressed as fold change. Because all real-time PCR data show the same direction in changes in gene expression profiles, we assume that the microarray data provide valid gene expression profiles for all gene reporters. We investigated the effects of pooling different samples on the outcome of the gene expression analysis by running real-time PCR analyses on seven genes in all individual samples and subsequently comparing those results with the results from the pooled samples. We showed that the results from the pooled samples represented unidirectional changes in almost all cases. Only the real-time PCR data from one gene, measured in one volunteer, were not in agreement with the results observed in the pooled samples. Overall, the pooling strategy seemed a successful approach as representation of individual responses in most gene reporter analyses.

It was not possible to establish the functional consequences of the regulation of the processes identified in Table 3. The impact of the iron interventions on gene expression profiles differed between the two pools, owing to biological, interindividual variation. Future studies to the functional consequences of changes in the gene expression profiles, as identified in this study, will have to be designed to determine the biological impact of the present study.

This study showed that iron administration in the human small intestine induced lipid peroxidation, and a rise in antioxidant capacity of the small intestinal secretions, in a dose-dependent fashion. Both iron dosages mediated the expression of many gene reporters, indicating that the novel approach to study nutrient-gut interactions was successful to detect many transcriptional changes. Hence, the applied technique offers a unique method to study effects of a single dietary component on transcriptome profiles in healthy individuals as well as in patients. Besides considerable inter- and intra-individual differences in gene expression profiles, we identified six processes that appeared to be strongly and consistently regulated by iron administration or iron-induced oxidative stress in the small intestine. Further studies are needed to establish the functional consequences of acute and chronic iron administration and oxidative stress in the human small intestine.

GRANTS

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REFERENCES

1. Aghdassi E, Carrier J, Cullen J, Tischler M, and Allard JP. Effect of iron supplementation on oxidative stress and intestinal inflammation in rats with acute colitis. *Dig Dis Sci* 46: 1088–1094, 2001.
2. Andoh A, Fujiyama Y, Bamba T, and Hosoda S. Differential cytokine regulation of complement C3, C4, and factor B synthesis in human intestinal epithelial cell line, Caco-2. *J Immunol* 151: 4239–4247, 1993.
3. Berg van den R, Haenen G, van den Berg H, and Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry*: 511–517, 1999.
4. Birkenkamp-Demtroder K, Christensen LL, Olesen SH, Frederiksen CM, Laiho P, Aaltonen LA, Laurberg S, Sorensen FB, Hagemann R, and ORntoft TF. Gene expression in colorectal cancer. *Cancer Res* 62: 4352–4363, 2002.
5. Boeckmann B, Bairoch A, Apweiler R, Blatter MC, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O'Donovan C, Phan I, Pilbout S, and Schneider M. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Res* 31: 365–370, 2003.
6. Consortium TGO. Creating the gene ontology resource: design and implementation. *Genome Res* 11: 1425–1433, 2001.

7. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, and Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat Genet* 31: 19–20, 2002.
8. Dupic F, Fruchon S, Bensaid M, Loreal O, Brissot P, Borot N, Roth MP, and Coppin H. Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice. *Gut* 51: 648–653, 2002.
9. Fodor I and Marx JJ. Lipid peroxidation of rabbit small intestinal microvillus membrane vesicles by iron complexes. *Biochim Biophys Acta* 961: 96–102, 1988.
10. Frazer DM, Wilkins SJ, Becker EM, Murphy TL, Vulpe CD, McKie AT, and Anderson GJ. A rapid decrease in the expression of DMT1 and Dcytb but not Ireg1 or hephaestin explains the mucosal block phenomenon of iron absorption. *Gut* 52: 340–346, 2003.
11. Geerling BJ, Badart-Smook A, Stockbrügger RW, and Brummer RJ. Comprehensive nutritional status in patients with long-standing Crohn disease currently in remission. *Am J Clin Nutr* 67: 919–926, 1998.
12. Gerhold D, Lu M, Xu J, Austin C, Caskey CT, and Rushmore T. Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays. *Physiol Genomics* 5: 161–170, 2001.
13. Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev* 21: 90–113, 2000.
14. Gonzalez PK, Doctrow SR, Malfroy B, and Fink MP. Role of oxidant stress and iron delocalization in acidosis-induced intestinal epithelial hyperpermeability. *Shock* 8: 108–114, 1997.
15. Lih-Brody L, Powell SR, Collier KP, Reddy GM, Cerchia R, Kahn E, Weissman GS, Katz S, Floyd RA, McKinley MJ, Fisher SE, and Mullin GE. Increased oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 41: 2078–2086, 1996.
16. Marx JJ and van Asbeck BS. Use of iron chelators in preventing hydroxyl radical damage: adult respiratory distress syndrome as an experimental model for the pathophysiology and treatment of oxygen-radical-mediated tissue damage. *Acta Haematol* 95: 49–62, 1996.
17. Odetti P, Valentini S, Aragno I, Garibaldi S, Pronzato MA, Rolandi E, and Barreca T. Oxidative stress in subjects affected by celiac disease. *Free Radic Res* 29: 17–24, 1998.
18. Rao L, Puschnier B, and Prolla TA. Gene expression profiling of low selenium status in the mouse intestine: transcriptional activation of genes linked to DNA damage, cell cycle control and oxidative stress. *J Nutr* 131: 3175–3181, 2001.
19. Reifen R, Matas Z, Zeidel L, Berkovitch Z, and Bujanover Y. Iron supplementation may aggravate inflammatory status of colitis in a rat model. *Dig Dis Sci* 45: 394–397, 2000.
20. Siegers CP, Bumann D, Trepkau HD, Schadwinkel B, and Baretton G. Influence of dietary iron overload on cell proliferation and intestinal tumorigenesis in mice. *Cancer Lett* 65: 245–249, 1992.
21. Srigiridhar K and Nair KM. Iron-deficient intestine is more susceptible to peroxidative damage during iron supplementation in rats. *Free Radic Biol Med* 25: 660–665, 1998.
22. Srigiridhar K, Nair KM, Subramanian R, and Singotamu L. Oral repletion of iron induces free radical mediated alterations in the gastrointestinal tract of rat. *Mol Cell Biochem* 219: 91–98, 2001.
23. Thykjaer T, Workman C, Kruhøffer M, Demtröder K, Wolf H, Andersen LD, Frederiksen CM, Knudsen S, and Ørntoft TF. Identification of gene expression patterns in superficial and invasive human bladder cancer. *Cancer Res* 61: 2492–2499, 2001.
24. Troost F, Saris W, Haenen G, Bast A, and Brummer RJ. A new method to study oxidative damage and antioxidants in the human small bowel: effects of iron application. *Am J Physiol Gastrointest Liver Physiol* 285: G354–G359, 2003.
25. Ueki T, Mizuno M, Uesu T, Kiso T, Nasu J, Inaba T, Kihara Y, Matsuoaka Y, Okada H, Fujita T, and Tsuji T. Distribution of activated complement, C3b, and its degraded fragments, iC3b/C3dg, in the colonic mucosa of ulcerative colitis (UC). *Clin Exp Immunol* 104: 286–292, 1996.
26. Wurzelmann JI, Silver A, Schreinemachers DM, Sandler RS, and Everson RB. Iron intake and the risk of colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 5: 503–507, 1996.
27. Xiao J, Gregersen S, Kruhøffer M, Pedersen SB, Ørntoft TF, and Hermansen K. The effect of chronic exposure to fatty acids on gene expression in clonal insulin-producing cells: studies using high density oligonucleotide microarray. *Endocrinology* 142: 4777–4784, 2001.
28. Yamada T and Grisham MB. Role of neutrophil-derived oxidants in the pathogenesis of intestinal inflammation. *Klin Wochenschr* 69: 988–994, 1991.
29. Zoller H, Koch RO, Theurl I, Obrist P, Pietrangelo A, Montosi G, Haile DJ, Vogel W, and Weiss G. Expression of the duodenal iron transporters divalent-metal transporter 1 and ferroportin 1 in iron deficiency and iron overload. *Gastroenterology* 120: 1412–1419, 2001.