Induction of arachidonate 12-lipoxygenase (Alox15) in intestine of iron-deficient rats correlates with the production of biologically active lipid mediators

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Submitted 15 June 2007; accepted in final form 4 February 2008

Collins JF, Hu Z, Ranganathan PN, Feng D, Garrick LM, Garrick MD, Browne RW. Induction of arachidonate 12-lipoxygenase (Alox15) in intestine of iron-deficient rats correlates with the production of biologically active lipid mediators. Am J Physiol Gastrointest Liver Physiol 294: G948–G962, 2008. First published February 7, 2008; doi:10.1152/ajpgi.00274.2007.—To identify novel genes associated with iron metabolism, we performed gene chip studies in two models of iron deficiency: iron-deprived rats and rats deficient in the principal intestinal iron transporter, divalent metal transporter 1 (i.e., Belgrade rats). Affymetrix rat genome gene chips were utilized (RAE230) with cRNA samples derived from duodenum and jejunum of experimental and control animals. Computational analysis and statistical data reduction identified 29 candidate genes, which were induced in both models of iron deficiency. Gene ontology analysis showed enrichment for genes related to lipid homeostasis, and one gene related to this physiological process, a leukocyte type, arachidonate 12-lipoxygenase (Alox15), was selected for further examination. TaqMan real-time PCR studies demonstrated strong induction of Alox15 throughout the small and large intestine, and in the liver of iron-deficient rats. Polyclonal antibodies were developed and utilized to demonstrate that proteins levels are significantly increased in the intestinal epithelium of iron-deprived rats. HPLC analysis revealed altered intestinal lipid metabolism indicative of Alox15 activity, which resulted in the production of biologically active lipid molecules (12-HETE, 13-HODE, and 13-HOTE). The overall effect is a perturbation of intestinal lipid homeostasis, which results in the production of lipids essentially absent in the intestine of control rats. We have thus provided mechanistic insight into the alteration in lipid metabolism that occurs during iron deficiency, in that induction of Alox15 mRNA expression may be the primary event. The resulting lipid mediators may be related to documented alterations in villus structure and cell proliferation rates in iron deficiency, or to structural alterations in membrane lipid composition.

duodenum; jejunum; microarray; arachidonic acid; Belgrade rat

Proper maintenance of body iron homeostasis is critical to avoid the pathological consequences of iron deficiency or iron overload, both described in detail over the past several years (2, 12, 18). Recent evidence has demonstrated that the hepatic peptide hormone hepcidin controls iron flux by regulating intestinal absorption from the diet and iron release from storage sites including the liver and reticuloendothelial (RE) macrophages (21, 22). This is likely accomplished by direct interaction of hepcidin with the iron exporter ferroportin (Fpn1) in these three key tissues, leading to internalization and degradation of Fpn1 (34). Hepcidin expression increases during states of iron overload and decreases during iron deficiency. The net results are to inhibit iron absorption from the diet and to sequester iron in RE macrophages and in the liver during iron overload and to enhance dietary absorption and iron release into the circulation from storage depots during deficient states. During systemic iron deficiency, decreased hepcidin expression thus allows normal activity of Fpn1 to export iron from enterocytes in the proximal small bowel into the interstitial fluid. Further regulatory mechanisms exist during iron deficiency that lead to the induction of several genes related to iron absorption including Fpn1, divalent metal transporter 1 (Dmt1; the brush-border iron intake protein), cytochrome b reductase 1 (Cybrd1; a protein involved in reduction of dietary iron), and transferrin receptor 1 (TfR1), which allows cells to uptake iron-loaded transferrin.

Utilizing genome scale expression profiling, we previously identified novel genes that were induced in the rat duodenum and jejunum by dietary iron deprivation (9, 11). Two mechanisms exist by which mRNA levels of iron homeostasis-related genes are regulated: one related to post-transcriptional mechanisms mediated by the iron response element/iron regulatory protein system (14, 37) and the other likely related to transcriptional regulation (8, 32). In either case, steady-state mRNA levels are altered and thus Affymetrix gene chips were able to detect the resulting alterations in gene expression. Our studies demonstrated strong induction of Dmt1, Cybrd1, TfR1, and Fpn1, along with a host of novel genes. These previous studies utilized nonstatistical methods to identify differentially expressed genes (DEGs), with a focus on genes that were highly expressed and regulated across several stages of postnatal development. It was interesting to note the novel genes that were identified utilizing this approach in that their regulation was similar to known iron transport-related genes, suggesting that some of them may indeed encode proteins that are important for iron homeostasis. One candidate gene, the Menkes copper ATPase (Atp7a), was further studied.

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report that epithelial cell differentiation and apoptosis (27). We now lipid homeostasis. Alox15 is involved in arachidonic acid EXPERIMENTAL PROCEDURES

representing many known iron-responsive genes such as Dmt1, Cybrd1, Tjfr1, heme oxygenase (Hmox1), and aminolevulinic acid synthase 1 (Alas1), along with novel genes related to copper homeostasis [e.g., Atp7a and metallothionein 1 and 2 (Mt1, Mt2)]. We performed additional genomic profiling studies with intestinal samples from a genetic model of iron deficiency, namely the Belgrade rat (gene symbol b), which has a point mutation in the Dmt1 gene that severely diminishes iron ion transport activity (16, 28). Analysis of the Belgrade rat data revealed several hundred DEGs in the duodenal and jejunal mucosa, with surprisingly few in common with the 1,483-DEG list from the rats deprived of dietary iron.

We were particularly interested in genes that were induced in both the dietary and genetic iron deficiency models. Focusing on the overlap between the two models allowed us to condense the Affymetrix gene chip data from 28,000 rats genes analyzed across 10 experimental groups to 29 genes considered strong candidates for further study. Contained within this list were Dmt1, Cybrd1, Fpn1, Tjfr1, Atp7a, Mt1, and Mt2. We subsequently performed gene ontology (GO) analysis to identify statistically overrepresented functional groups of genes in our experimental data. Interestingly, all data sets were enriched for functions related to lipid homeostasis. We also noted that the most strongly induced gene (over 80-fold in the b/b rats) was arachidonate 12-lipoxygenase (Alox15), itself related to lipid homeostasis. Alox15 is involved in arachidonic acid metabolism and may play a role in regulation of intestinal epithelial cell differentiation and apoptosis (27). We now report that Alox15 is induced up to >100-fold throughout the entire length of the intestine and in the liver of iron-deficient and Belgrade rats. Alox15 protein expression is also increased during iron deficiency. We further detected perturbations in intestinal lipid homeostasis consistent with induction of Alox15 enzyme activity in the gut of iron-deficient rats. We have thus progressed from observations made by genomewide expression profiling to concomitant metabolic changes in the intestine of a living mammal.

EXPERIMENTAL PROCEDURES

Experimental animals. Sprague-Dawley rats that were utilized for gene chip analyses were made iron deficient at different postnatal ages by dietary iron deprivation (11). These rats were studied at the following ages: 8 days (sucklings), 21 days (weanlings), 6 wk (adolescents), and 12 and 36 wk (adults). Belgrade rats used in the present studies were adult males between the ages of 6 and 14 mo, obtained from a breeding colony at the University at Buffalo maintained by Dr. Laura Garrick. The +/b rats were fed a normal chow, whereas the b/b rats were fed a high-iron-containing chow, per the usual husbandry routine (23). Three different groups of +/b and b/b rats (3 per genotype) were euthanized at different times, and duodenal and jejunal scrapings and livers were taken and snap frozen in liquid nitrogen. RNA was isolated by Trizol reagent followed by further purification with the RNEasy Mini Kit (Qiagen). RNA was quantified by UV spectrophotometry, and concentrations were normalized by agarose gel electrophoresis followed by optical density analysis on a Gel Doc (Bio-Rad).

Additional rats were utilized in the present studies for real-time PCR studies. Sprague-Dawley male rats were obtained from Harlan at 3 wk of age and were initially placed on a control diet (198 ppm Fe, 6 ppm Cu) or a low-iron diet (3 ppm Fe, 6 ppm Cu) for 2 wk. Then, half of the rats on the low-iron diet were switched to a diet deficient in iron and copper (3 ppm Fe, 0.3 ppm Cu); rats remained on respective diets for an additional 7 wk and were euthanized at 12 wk of age. All diets were identical except for the iron and copper content (Dyets; Bethlehem, PA). Blood was drawn by cardiac puncture after CO2 narcosis. Complete blood cell count analysis, serum copper, and liver copper and iron levels were determined by the Colorado Veterinary Diagnostic Laboratory at Colorado State University (Fort Collins, CO).

Gene chip studies. The methods utilized for the expression profiling with the rats from the dietary deficiency model at different ages were described in detail previously (11). The Belgrade RNA samples were processed and the gene chips run in identical fashion, which was essentially according to the manufacturer’s instructions (Affymetrix), utilizing their suggested reagents with RAE230 2.0+ arrays, representing 28,000 rat transcripts. The experiment was repeated three times with RNA samples isolated from three different groups of littermate +/b and b/b rats. Gene chip data have been submitted to the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo); data from the dietary deficiency groups can be found under accession no. GSE1892 and data from the Belgrade rat studies under accession no. GSE7267.

Gene chip data processing and analysis. Previous analysis of gene chip data (11) from the dietary deficiency rats was performed with a nonstatistical approach utilizing the MAS probe set algorithm and the Data Mining Tool software (Affymetrix). We have now employed the probe set algorithm RMA (26) to generate expression summary values, which represent the relative expression levels of genes from their corresponding probes, for the 72 Affymetrix Genechips (RAE230A and RAE230B chips representing triplicate experiments from the duodenum of the dietary-deficient rats at the five different ages and jejumum of the 12-wk-old rat group, control and iron deficient for each). We utilized the default RMA function included in the “Affy” package of Bioconductor in the R statistical computing environment (http://www.r-project.org/). This default function employs median polish for expression summary and quantile normalization for data normalization. We also used MASS.0 “present calls” to filter out probe sets whose expression intensities were close to the background noise across the majority of the samples before performing the differential gene analysis. We applied the filtering of at least two “present calls” out of three replicated samples in either the normal or iron deficiency rat group. This led to a 52.5–57.4% data reduction for the comparisons. To detect gene expression values significantly different between groups, we employed SAM (44) software by controlling the false discovery rate <4% and expression fold changes >1.5.

For clustering analysis, we selected 1,483 probe sets whose expression values were significantly different between controls and iron-deficient rats in at least one of the six experimental groups. We built a 1,483 × 6 matrix of average fold change between iron deficiency and control rat groups as input for clustering analysis. We then used K-mean clustering with the number of clusters set at three. Belgrade rat gene chip studies. Three groups of 3 +/b and 3 b/b adult rats (ages 6–14 mo) were obtained over the course of several months. Duodenal and jejunal mucosa were harvested, and RNA was purified and enzymatically converted to cRNA. Samples were subsequently hybridized to Rat Genome 230 2.0+ gene chips (Affymetrix; n = 3), representing over 28,000 rat transcripts. Chips were processed by standard methods. The MASS.0 statistical algorithm (Affymetrix) was used for the detection of DEGs. Data were filtered using the MASS.0 present call. The cutoff was set up to include at least two present calls out of three replicated samples in either control or iron
deficiency groups, filtering duodenum and jejunum separately, which removed 12,644 probe sets for jejunum and 12,915 for duodenum. Data were tested by regularized t-test (3), and genes with individual \( P \) values <0.05 were selected.

**GO analysis.** Resulting gene chip data were analyzed by the David 2.1 software (DAVID Bioinformatics Resources 2007, National Institute of Allergy and Infectious Diseases; http://david.abcc.nig.acrift.gov/). Data were submitted as either Affymetrix probe set ID or as Official Gene Symbol; both approaches resulted in highly similar results. Data were also analyzed by the NetAffx Gene Ontology Mining Tool available on the Affymetrix web site (www.affymetrix.com).

**Real-time PCR analysis.** RNA was purified from mucosal scrapings with Trizol reagent (Invitrogen) and quantified by UV spectrophotometry. RNA samples were either fresh or stored in ethanol at \(-80^\circ\)C until use. One microgram of RNA was reverse transcribed with a constitutive control gene, the RT reaction was diluted 1:100, and 1 \( \mu \)l was utilized in a 20-\( \mu \)l reaction with 5 \( \mu \)l of iQ supermix (Bio-Rad), 2 \( \mu \)l TaqMan primer/probe mix (Applied Biosystems) and nuclease-free water. For analysis of Alox15 (Applied Biosystems; assay ID Rn00696615_m1; target GenBank accession no. NM_031010), 2 \( \mu \)l of undiluted RT reaction was used. Reactions were run in 96-well plates on a Bio-Rad iCycler with the following cycling parameters: 50°C for 2 min, 95°C for 8.5 min, and 42 cycles with 95°C for 30 s and 60°C for 1 min. Each RT reaction was analyzed in duplicate for both 18S rRNA and Alox15 in each experiment. Then the 18S average was subtracted from the Alox15 average to generate the cycle threshold (\( C_t \)) value. Data were analyzed by routine methods. Briefly, \( \Delta \Delta C_t \) values from each gut segment were calculated from Alox15 and 18S rRNA \( C_t \) for iron- or iron/copper-deficient groups vs. the control groups. The \( \Delta \Delta C_t \) was the exponent of 2 for mean fold induction; its standard deviation was the exponent of 2 as an estimate of the range. Statistical analyses were done by t-test.

Alox15 antibody development. Polyclonal antibodies against the rat Alox15 protein (GenBank Locus ID AAA64930) were generated by injecting two rabbits with the following peptides: KTDKAVQD-DYELQSWCRE (amino acids 485–502), and KAVLKKFREEL-with argol and stored at \(-80^\circ\)C. For analysis of Alox15 (Applied Biosystems; assay ID Rn00696615_m1; target GenBank accession no. NM_031010), 2 \( \mu \)l of undiluted RT reaction was used. Reactions were run in 96-well plates on a Bio-Rad iCycler with the following cycling parameters: 50°C for 2 min, 95°C for 8.5 min, and 42 cycles with 95°C for 30 s and 60°C for 1 min. Each RT reaction was analyzed in duplicate for both 18S rRNA and Alox15 in each experiment. Then the 18S average was subtracted from the Alox15 average to generate the cycle threshold (\( C_t \)) value. Data were analyzed by routine methods. Briefly, \( \Delta \Delta C_t \) values from each gut segment were calculated from Alox15 and 18S rRNA \( C_t \) for iron- or iron/copper-deficient groups vs. the control groups. The \( \Delta \Delta C_t \) was the exponent of 2 for mean fold induction; its standard deviation was the exponent of 2 as an estimate of the range. Statistical analyses were done by t-test.

Intestinal sample preparation. Samples were prepared for Western blotting and lipid analysis as follows, with all steps being performed at 4°C. Intestinal mucosa scrapings from duodenum and jejunum of control and iron-deficient rats were homogenized in \(-4–5 \mu l\) sample buffer 1 (0.05 M Tris-HCl, pH 7.4 at 22°C, 0.05 M NaCl, 0.001 M EDTA, + protease inhibitor cocktail) per gram of tissue with a tissue grinder (model 85370-395, Biospec products, Bartlesville, OK) at setting “20” for 2 min, and centrifuged at 16,000 g for 15 min. The supernatant was re-centrifuged at 110,000 g for 1 h. This supernatant was termed “cytosol.” The resulting pellet was resuspended in sample buffer containing 0.25% (vol/vol) Tween 20 (buffer 2), sonicated for 20 s in water-ice slush twice, in a Vibra cell (Sonics and Materials, Danbury, CT), at output of \(-40\%\) intensity with 1 min of chilling in between, and then centrifuged at 16,000 g for 30 min. This supernatant equates solubilized particulate membrane fraction. For total lysates, the tissues were directly homogenized in buffer 2, whereas all other steps remained the same. Samples were adjusted to 20% glycerol and stored at \(-20^\circ\)C until use.

Immunoprecipitation. All steps were performed at 4°C. Comparability of samples [by BCA assay (Pierce, Rockford, IL) using BSA as standards] from control and iron-deficient rat intestinal tissue in sample buffer 2 were immunoprecipitated with 1:4,000 dilution of rabbit polyclonal anti-rat Alox15 antiserum in a final volume of 500 \( \mu l \) by gentle mixing overnight. A 50% slurry (30 \( \mu l \)) of protein A-Sepharose was added and mixed for 1 h. The ternary complex was centrifuged at 3,000 g for 3 min, and the flow-through fraction was saved. Pellets were washed with 500 \( \mu l \) of the sample preparation buffer three times. Samples were then run on denaturing gels and the gels were subsequently stained with silver stain (Invitrogen). Purified Alox15 enzyme was obtained from Cayman Chemical (Ann Arbor, MI).

**SDS-PAGE and Western blotting.** Identical quantities of protein from control and iron-deficient rat intestinal tissue in sample buffer and/or the washed pellets from the IP reaction were solubilized in reducing sample buffer, heated to 70°C for 10 min, and subjected to reducing SDS-10% PAGE according to manufacturer’s recommendations (Invitrogen; Carlsbad, CA). The gel-resolved samples were electrobotted onto polyvinylidene difluoride (PVDF) membranes (Millipore; Bedford, MA) following the manufacturer’s recommendations (Invitrogen).

**Immunopроб.** The PVDF blot was blocked with blocking buffer [5% nonfat dry milk in TBST: 0.05 M Tris-HCl, pH 7.4 at 22°C, 0.15 M NaCl, 0.05% (vol/vol) Tween 20] for 90 min and then reacted with a 1:4,000 dilution of anti-Alox15 antiserum in blocking buffer for 1.5 h. Subsequently, three washes of 5, 7, and 10 min were performed in TBST, followed by reacting with a 1:5,000 dilution of horseradish peroxidase-conjugated anti-rabbit (secondary) antibody in blocking buffer, followed by three more washes as stated above. For peptide blocking experiments, the antiserum was incubated with an equal volume of both peptides (1 mg/ml for each) followed by incubation at room temperature for 1–2 h. The immune serum was also incubated at room temperature with an equal volume of PBS. The blot was exposed to the substrate (\( H_2O_2 \))-enhancer mixture SuperSignal Chemiluminescent Substrates for Western blotting (Pierce; Rockford, IL) and exposed to X-ray film (Phenix Research Products, Candler, NC) per the manufacturer’s recommendations. The blot was subsequently stained with Ponceau S for protein quantitation with a Bio-Rad LS750 scanning densitometer and Quantity One software.

**Immunohistochemical analysis of intestinal tissue from control and iron-deficient rats.** Tissue was harvested and processed as described below under Histopathological analysis of proximal small intestine. Parafin sections on slides were deparaffinized with xylene and a series of ethanol washes, essentially as previously described in detail (10). Sections were then blocked for 30–45 min with immunohistochemistry (IHC) blocking solution (Bethyl Laboratories) followed by a 10-min wash in PBS. The Alox15 polyclonal antiserum was then applied at a 1:500 dilution overnight in a humidified chamber, followed by a 10-min wash in PBS. Peptide blocking was performed as described above. A secondary antibody (Alexa Fluor 647 goat anti-rabbit IgG; Invitrogen Molecular Probes) was then applied for 30 min at a 1:1,000 dilution overnight in a humidified chamber, followed by a 10-min wash in PBS. The next day, the section was counterstained with DAPI and mounted with a fluorescent mounting medium. Slides were visualized in the Confocal and Flow Cytometry Facility in the School of Medicine and Biomedical Sciences at the University of Buffalo with a Zeiss LSM 510 Meta Confocal scanhead mounted to an Axiovert 200 M inverted fluorescent microscope. A 633-nm line from a HeNe laser was used for sample excitation along with a Cy5 emission filter set. The confocal settings were kept identical across the different samples, so direct comparison of fluorescence intensity is possible.

**Intestinal lipid analysis.** Intestinal samples were initially processed as follows, for experiments 1 and 2. Animals were euthanized and mucosal scrapings were ground with a tissue homogenizer in a Tris buffer (0.05 M; pH 7.4). Samples were then centrifuged at 16,000 g for 10 min at 4°C and the supernatant was collected for HPLC studies. Subsequent experiments (numbers 3–6) utilized additional sample preparation methods described above under Intestinal sample preparation. In some cases, samples were incubated at 37°C with 100 \( \mu M \) arachidonic acid for 30 min prior to lipid analysis. One volume of tissue homogenate or cytosol or membrane preparation was mixed
with one volume of absolute ethanol and centrifuged at 10,000 g for 15 min. The cleared supernatant was removed for HPLC analysis. Fatty acids and their hydroperoxo and hydroxy products were determined simultaneously by HPLC with diode array detection (6). HPLC instrumentation consisted of a Shimadzu LC-10AVp pump, a FCV 10 flow-control valve, a DGU14 degassing unit, a SPD-M10A photodiode array, a SIL-7A autosampler, and an IBM PC equipped with EZ Start chromatography data software (Shimadzu Scientific Instruments, Columbia, MD). All solvents were HPLC grade from Fisher Scientific (Fair Lawn, NJ). Peroxide-free linoleic, linolenic, and arachidonic acids and 13-hydroxy-octadecatrienoic acid (13-HODE), 12-hydroxy-eicosatetraenoic acid (12-HETE), and other lipid standards were purchased from Cayman Chemical (Ann Arbor, MI) (6). For analysis, 150 μl of the prepared sample was injected onto a SupelcoSil LC-18 (25 cm × 4.6 mm ID, 5-μm particle size, Supelco, Bellefonte, NJ) reversed-phase column and eluted isocratically with a mobile phase of acetonitrile:0.1% acetic acid-tetrahydrofuran (41:41:4 vol/vol/vol) at a flow rate of 0.1 ml/min. The column eluant was monitored by the photodiode array from 200–300 nm. Hydroxy and hydroperoxy fatty acids were quantified at 234 nm and native fatty acids were quantified at 215 nm.

RESULTS

Experimental animals. Rats that were deprived of dietary iron displayed signs of microcytic, hypochromic anemia, with the exception of the 36-wk-old rats (11). The 36-wk-old rats were not deprived of iron until they were sexually mature adults, and hence iron recycling is very efficient and dietary needs are greatly diminished. The homozygous Belgrade rats (bl/bl) were also anemic, whereas the heterozygotes (+/bls) were phenotypically normal (23). Blood and metals analysis was also done on rat groups utilized for quantitative RT-PCR (qRT-PCR) and lipid analysis (Table 1); iron- and iron/copper-deprived rats were anemic. Moreover, serum and liver copper levels were significantly increased in iron-deficient rats, in agreement with our previous observations (36), whereas serum and liver copper was decreased in iron/copper-deficient rats (all compared with controls). Liver iron levels were decreased to a similar extent in both deficiency groups.

**Table 1. Rat blood analysis and metal analysis of liver and serum**

<table>
<thead>
<tr>
<th>CBC Analysis</th>
<th>Hct (n = 3)</th>
<th>P Value</th>
<th>RBC (n = 6)</th>
<th>P Value</th>
<th>HGB (n = 6)</th>
<th>P Value</th>
<th>PCV (n = 6)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.40 ± 1.90</td>
<td>&lt;0.0001</td>
<td>7.97 ± 1.14</td>
<td>&lt;0.0001</td>
<td>14.76 ± 0.42</td>
<td>&lt;0.0001</td>
<td>46.40 ± 1.14</td>
<td>&lt;0.0001</td>
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<tr>
<td>Fe deficient</td>
<td>9.77 ± 0.78*</td>
<td>0.0001</td>
<td>3.82 ± 0.37*</td>
<td>0.0001</td>
<td>2.46 ± 0.21*</td>
<td>0.0001</td>
<td>13.4 ± 0.80*</td>
<td>0.0001</td>
</tr>
<tr>
<td>Fe/Cu deficient</td>
<td>6.27 ± 1.97</td>
<td>&lt;0.0001</td>
<td>2.52 ± 0.65</td>
<td>&lt;0.0001</td>
<td>1.60 ± 0.38</td>
<td>&lt;0.0001</td>
<td>8.17 ± 2.04</td>
<td>0.0001</td>
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<table>
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<tr>
<th>MCV (n = 5)</th>
<th>P Value</th>
<th>MCHC (n = 5)</th>
<th>P Value</th>
<th>RDW (n = 5)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56.00 ± 4.74</td>
<td>&lt;0.0001</td>
<td>31.80 ± 0.45</td>
<td>&lt;0.0001</td>
<td>12.08 ± 0.18</td>
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<tr>
<td>Fe deficient</td>
<td>35.0 ± 1.67</td>
<td>&lt;0.0001</td>
<td>18.40 ± 0.49</td>
<td>&lt;0.0001</td>
<td>27.50 ± 1.66</td>
</tr>
<tr>
<td>Fe/Cu deficient</td>
<td>32.67 ± 3.67</td>
<td>&lt;0.0001</td>
<td>20.00 ± 2.45</td>
<td>&lt;0.0001</td>
<td>28.83 ± 2.65</td>
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<table>
<thead>
<tr>
<th>Liver Cu (n = 8)</th>
<th>P Value</th>
<th>Serum Cu (n = 8)</th>
<th>P Value</th>
<th>Liver Fe (n = 8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.1 ± 2.1</td>
<td>&lt;0.0001</td>
<td>0.5 ± 0.08</td>
<td>&lt;0.0001</td>
<td>669.0 ± 50.6</td>
</tr>
<tr>
<td>Fe deficient</td>
<td>72.2 ± 6.3*</td>
<td>&lt;0.0001</td>
<td>0.76 ± 0.10*</td>
<td>&lt;0.0001</td>
<td>197.2 ± 23.6</td>
</tr>
<tr>
<td>Fe/Cu deficient</td>
<td>5.69 ± 1.7</td>
<td>&lt;0.0001</td>
<td>0.03 ± 0.02</td>
<td>&lt;0.0001</td>
<td>215.8 ± 46.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. Hct, hematocrit (%); RBC, red blood count (× 10⁶/μl); HGB, hemoglobin (g/dl); PCV, packed cell volume (%); CBC, complete blood cell count; MCV, mean corpuscular volume (fL); MCHC, mean cell hemoglobin concentration (g/dl); RDW, red cell distribution width; Cu and Fe analysis, all in parts/million (ppm). *P ≤ 0.05 Fe-deficient vs. Fe/Cu-deficient rats.

AJP-Gastrointest Liver Physiol • VOL 294 • APRIL 2008 • www.ajpgi.org
duodenum (Supplemental Table S2) and 515 DEGs in jejunum (Supplemental Table S3). Interestingly, comparison of all Belgrade DEGs (818 total) to the 1,483 DEGs from the dietary deficiency groups revealed only 109 DEGs in common (not shown). Furthermore, when the total data set from the Belgrades was compared with the 12-wk-old iron-deficient rat data set (i.e., the group that was most similar to the Belgrades in regard to the fact that they were adults that were iron-deficient from an early postnatal age), only 55 DEGs were in common (not shown). Both of these groups of overlapping DEGs were
also statistically enriched for biological processes related to lipid homeostasis and iron metabolism. Our final analysis generated a list of 29 DEGs that were in common between the 228 upregulated cluster from the 1,483 list and the Belgrade increasers; GO analysis showed similar enrichments as in all previously mentioned analyses (Fig. 2). This subset of DEGs included iron homeostasis-related genes (Dmt1, Cybrd1, Fpn1, and Tfr1), genes related to copper homeostasis (Atp7a, Mt1, Mt2), and novel genes associated with lipid homeostasis (Gpam, Ldhd, Pc, Scd, and Alox15) (Table 2).

Real-time PCR. Very strong induction of Alox15 was detected in all samples studied (Fig. 3). Our experimental groups included adult rats that had been deprived of iron since weaning, adult $+/b$ and $bb$ rats, and rats that had combined iron/copper deficiency. We detected induction in gut segments from duodenum to distal colon and also in liver.

Western blot and immunohistochemical analyses. We detected two prominent protein bands on Western blots of $72$ kDa from three separate groups of rats (Fig. 4A). The bands were much stronger in the iron-deficient groups compared with controls (average $\sim 4$-fold; Fig. 4B). Since we were unable to consistently detect $\beta$-actin protein in the iron-deficient groups, we performed optical density analysis of total protein in each lane of the stained blot (Fig. 4A, right) to normalize Alox15 protein expression. Additional experiments were performed to determine whether the antiserum was specific for Alox15. Reaction of blots with preimmune serum from the same rabbit showed no protein bands (data not shown), whereas peptide blocking significantly reduced the antigenic signal ($\sim 14$-fold; Fig. 4C), providing evidence that the antiserum was indeed recognizing the Alox15 protein. Further confirmation came from IP experiments whereby we were able to pull down two protein bands from rat intestinal cytosolic and membrane preparations that were identical to the bands pulled down from a commercial Alox15 prep (Fig. 4D).
We next sought to localize the protein in the rat small intestine and colon utilizing fixed tissue sections from control and iron-deficient rats and the Alox15 antisera. Confocal microscopic imaging revealed strong protein expression in enterocytes along the crypt-villus axis in iron-deprived rats and the Alox15 antiserum. Confocal intestine and colon utilizing fixed tissue sections from control groups (Fig. 5), with stronger fluorescence again detected in the iron-deficient rats. Quantitative data revealed strong induction of 13-HODE only in jejunum (Fig. 7). Rats with combined iron and copper deficiency also exhibited increased levels of 12-HETE, 13-HOTE, and 13-HODE; in fact, these lipids were again undetectable or barely detectable in samples derived from control rats (Supplemental Table S4). Furthermore, the starting polyunsaturated fatty acids (PUFAs) were consistently not different between groups. When we analyzed the entire body of lipid analysis data from the six experiments, we again found identical results (Supplemental Table S4).

**Histopathological analysis of proximal small intestine.** Analysis of the morphology of the intestinal epithelium by a senior pathology resident revealed alterations in the iron-deficient group (Table 3). Villus height, crypt width, mucosal thickness, and the number of mitotic cells were all increased in the iron-deficient rats compared with controls.

**DISCUSSION.**

Genes encoding proteins related to intestinal nutrient absorption and homeostasis are often regulated by the dietary availability of the nutrient. For example, an intestinal phosphate transporter responds to dietary P depriviation (35), and intestinal folate transport is enhanced by dietary folate deprivation.
Genes related to iron metabolism were anticipated (1, 11, 33, 48), as were genes related to cell cycle control, because iron deprivation causes cells to undergo apoptosis (20, 24, 25, 30). What was surprising, however, was enrichment for genes related to lipid homeostasis, since we were unable to find published reports linking iron deficiency to alterations in lipid metabolism. Clustering analysis of the 1,483 DEGs identified a large group of genes that was induced by iron deficiency (i.e., 228 unique probe sets). This upregulated cluster of genes contained known iron-responsive genes such as Dmt1, Cybrd1, Tfrl, and Hmox1; it also contained many novel genes not previously associated with iron homeostasis. GO analysis of the 228 upregulated cluster showed similar results, with an enrichment of genes related to iron and lipid homeostasis.

Analysis of the gene chip data from the Belgrade rats identified several hundred DEGs; surprisingly, very few DEGs were in common with the dietary deficiency model. These findings may be related to the fact that the b/b rats are fed a high-iron-containing diet (300 ppm), whereas the iron-deficient rats had only 3 ppm iron in the diet (yet both have systemic iron deficiency). Both the +/b and control rats consumed diets containing around 200 ppm iron. Unique DEGs in either model could therefore reflect the differing amounts of iron present in the gastrointestinal tract. Furthermore, it is likely that the higher amount of iron present in the gut lumen of the b/b rats may result in oxidative stress and that high or low luminal iron may result in alterations in the composition of the bacterial flora. Both of these conditions would likely alter the expression of genes in the intestinal mucosa. We thus surmised that genes that were similarly induced in both models were likely to be primarily responsible for the adaptive response to iron deprivation. Utilizing this strategy, we could eliminate those DEGs related to the dietary level of iron and identify genes that were responding to iron deficiency per se. This analysis culminated in the identification of 29 genes as strong candidates for further study. Again, GO analysis of these genes revealed enrichment for genes related to lipid homeostasis.

This list of 29 DEGs resulted from significant data reduction strategem that allowed us to condense a large body of data from eight experimental groups (n = 3 for each) with Affymetrix gene chips representing over 28,000 rat genes to a manageable list of candidate genes. One gene of particular interest was Alox15 (Affymetrix probe set ID 1387796_at; target GenBank accession no. NM_031010). Alox15 drew our attention for three reasons: 1) Alox15 is involved in lipid homeostasis, 2) Alox15 was the most strongly induced gene in the entire body of gene chip data (over 80-fold in the Belgrade duodenum), and 3) Alox15 is a nonheme iron-containing protein (31). We confirmed induction of Alox15 by qRT-PCR with a specific TaqMan primer/probe set; Alox15 was strongly induced along the horizontal axis of the intestine from duodenum to distal colon and in the liver of iron and iron/copper-deficient rats, and in b/b rats.

We propose that the interactions depicted in Fig. 8 occur in the intestinal mucosa. What is not clear at this point is which of the possible physiological effects depicted in the figure may be in play in the iron-deficient gut. The following sections will describe our experimental findings that led to the development of this schema and will provide justification based on known intestinal physiology and lipid homeostasis.
If changes in mRNA expression are to have functional significance, then protein levels should also be altered. We thus generated a polyclonal antiserum against the rat Alox15 protein to quantify protein expression levels and to localize expression in the rat intestine. Western blot analysis revealed an increase in protein levels in the intestine of iron-deficient rats. Two distinct bands were detected, both of which were increased in iron deficiency and blockable with the antigenic peptides. The preimmune serum did not recognize these bands on Western blots. Moreover, IP of intestinal protein pulled down two bands that were identical to those pulled down in a commercial Alox15 prep. Protein from a group of control and iron-deficient rats was separated by SDS-PAGE and blotted onto nitrocellulose. A polyclonal antiserum against rat Alox15 that was preincubated with the 2 antigenic peptides (Blocked) or saline (Immune) was reacted with the blot. The images on the left are from the film and the right 2 images show the stained blot. D: immunoprecipitation (IP) of Alox15 protein. The Alox15-specific antiserum was utilized to perform IP with cytosolic or membrane protein from rat intestine or from a commercial Alox15 reagent (Alox151). Shown is the silver-stained gel. The approximate placement of molecular mass markers is shown at left.

Fig. 4. Western blot analysis of rat Alox15 protein expression. On the left side of A is shown the X-ray film indicating strong immunoreactive bands at ~72 kDa, consistent with the predicted size of the rat Alox15 protein. Each group was 2 control (C) or iron-deficient (L) adult rats (~12 wk of age) that had been on specialized diets since weaning. On the right side of A is shown the stained gel, which was scanned to determine the total protein in each lane. Molecular mass markers are shown at far right. Additional experiments with samples derived from other groups of rats showed similar results with greater protein expression in the iron-deficient group (data not shown). B: quantitation of Alox15 protein expression. The X-ray film was scanned as was the protein on the blot. Optical density data from the film were normalized for total protein amounts on the blot. Statistical analysis was by t-test. C: peptide blocking Western blot. Protein from a group of control and iron-deficient rats was separated by SDS-PAGE and blotted onto nitrocellulose. A polyclonal antiserum against rat Alox15 that was preincubated with the 2 antigenic peptides (Blocked) or saline (Immune) was reacted with the blot. The images on the left are from the film and the right 2 images show the stained blot. D: immunoprecipitation (IP) of Alox15 protein. The Alox15-specific antiserum was utilized to perform IP with cytosolic or membrane protein from rat intestine or from a commercial Alox15 reagent (Alox151). Shown is the silver-stained gel. The approximate placement of molecular mass markers is shown at left.

G956 INTESTINAL Alox15 INDUCTION DURING IRON DEFICIENCY

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other known enzyme could produce this profile of lipid products. We determined the identity of these lipids by HPLC comparison to lipid standards and by reduction of the samples with borohydride, which did not alter the elution spectrum of the products (not shown). These lipids were essentially absent on the HPLC plots from control rats, confirming a strong induction of Alox15 activity in the intestine of iron-deficient rats. Quantitative data showed

Fig. 5. Immunohistochemical analysis of Alox15 protein expression in rat duodenum and colon. Fixed tissue sections were reacted with the Alox15 specific antiserum (Immune) followed by a fluorescent-tagged secondary antibody and imaging with a confocal microscope. Alox15 protein is depicted by the red color. A: fluorescent images are overlaid on differential interference contrast (DIC) images of the tissue sections. Shown in the bottom 2 panels are iron-deficient (low-Fe) samples reacted with preimmune or peptide-blocked serum. B: Alox15 protein is depicted by the red color in these “cloth-cut” sections through the colonic crypts. Shown on the left are fluorescent images overlaid on DIC images of the sections. Shown on the right are the fluorescent images. The confocal settings remained constant across all images. Images are typical of several experiments that were performed.
strongest induction of 12-HETE and 13-HOTE, particularly in the jejunum, where 12-HETE was 30 times more abundant and 13-HODE was 80 times more abundant than in control rats.

Despite the strong correlation between Alox15 induction and altered lipid profiles consistent with its activity, other possibilities need to be considered. Lipid peroxidation can be enzymatically mediated, but it may also result from mass action or metal ion-mediated effects. Mass action effects would result if more starting PUFAs were present, because these lipids will spontaneously oxidize at low rates. We did not, however, detect increased amounts of arachidonic, linoleic, or linolenic acids in the samples from iron-deficient rats (compared with controls). We also considered the possibility that copper ion-mediated lipid peroxidation occurred, since we previously detected increased copper levels in the intestinal mucosa of iron-deficient rats (36). To address this issue, we generated rats that had combined iron and copper deficiency and performed additional studies. Alox15 mRNA expression was also strongly induced throughout the gut of these experimental rats, and there were again altered lipid profiles (similar to what was observed in iron-deficient rats). Furthermore, metal ion-mediated lipid peroxidation generally leads to mixtures of positional isomers of hydroperoxy and hydroxy PUFAs, whereas enzymatic lipid peroxidation processes generally lead to a single specific product (42). In the case of linoleic and linolenic acids, metal ion peroxidation leads to equimolar amounts of 9- and 13-regioisomers. In the case of arachidonic acid (eicosahexaenoic acid), 5-, 8-, 9-, 11-, 12-, and 15-regioisomeric products are possible (41). The presence of only 13-HOTE, 13-HODE, and 12-HETE strongly indicates an enzymatic mechanism. We conclude then that it is unlikely that metal ion-mediated lipid peroxidation occurs. These findings overall support the hypothesis that induction of Alox15 in the intestine of iron-deficient rats was harvested and processed for HPLC analysis. The samples were run along with known lipid standards for comparison. The time to elution from the column is indicative of the identity of a particular lipid. Lipid standards (top) suggest the identity of strong peaks detected in iron-deficient rats (middle), which were weak or absent in controls (bottom). mAu, milliabsorbance units; HOTE, hydroxy-octadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HpODE, hydroperoxy-octadecadienoic acid.

Fig. 6. Lipid analysis of intestinal mucosa. Intestinal mucosa from control and iron-deficient rats was harvested and processed for HPLC analysis. The samples were run along with known lipid standards for comparison. The time to elution from the column is indicative of the identity of a particular lipid. Lipid standards (top) suggest the identity of strong peaks detected in iron-deficient rats (middle), which were weak or absent in controls (bottom). mAu, milliabsorbance units; HOTE, hydroxy-octadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HpODE, hydroperoxy-octadecadienoic acid.
rats is the molecular event leading to the production of biologically active lipid mediators.

The molecular signal that leads to the induction of Alox15 during iron deficiency is unknown. It is clear that iron deficiency may produce significant pathological changes in tissues including altered activity of iron-dependent enzymes, hypoxia, and induction of apoptosis due to low cellular iron content (29). Our data suggest that Alox15 induction is related to increased gene transcription rates or to altered message stability. Interestingly, the Alox15 transcript has no detectable iron response element nor does its promoter have an experimentally verified hypoxia response element. Alox15 mRNA expression is regulated by various cytokines and growth factors and transcriptional regulation has been described in a number of studies (50). If these reported mechanisms are involved in the induction of Alox15 in the intestine and liver of iron-deficient rats remains to be determined. Moreover, it is possible that induction occurs to maintain enzyme levels for normal homeostasis, since Alox15 is an iron-dependent enzyme. Two lines of reasoning argue against this, however: 1) many iron-dependent enzymes were not induced by iron deficiency in our studies (e.g., catalase, myeloperoxidase, tryptophan dioxygenase), whereas others were downregulated (e.g., glycerol phosphate dehydrogenase), and 2) induction of Alox15 results in the robust production of lipids that are present either not at all or at very low levels in control rats.

Three isoforms of 12-lipoxygenases have been described, named after the cells where they were first identified: platelet, leukocyte, and epidermis (50). Existing terminology in the literature is confusing; the rodent, leukocyte-type, 12-lipoxygenase is called Alox15 (46, 47). Despite the name, the rodent Alox15 is in reality a 12-lipoxygenase that may also have lesser 15-lipoxygenase activity, although in our studies, as described below, we did not detect any 15-lipoxygenase products (i.e., 15-HETE). The leukocyte-type enzyme is widely distributed in different cell types, but tissue distribution varies substantially from species to species. The platelet and epidermal enzymes are present in only a relatively limited number of cell types. Our present data are thus consistent with detection of a leukocyte-type 12-lipoxygenase, since our profile of lipid products parallels the described activity of the purified enzyme from porcine leukocytes (49). Moreover, both the epidermal and platelet-type 12-lipoxygenases have very low activity against linoleic and linolenic acids (50). Of further interest is the fact that previous studies of leukocyte-type Alox15 enzymes isolated from different tissues/cell types did not produce the same pattern of products as we report here; for instance, it was reported that Alox15 produces 12- and 15-HETE in a 3:1 molar ratio (19) and we did not detect production of 15-HETE. The native Alox15 enzyme in the rat intestine may thus have unique properties.

The lipid products produced by Alox15 are considered biologically active lipid mediators (5); they may interact with cell surface receptors and induce signal transduction pathways or may interact with nuclear receptors such as the peroxisome proliferator-activated receptors (17) to regulate gene expression. In many cases, lipoxygenase-modulated events may only become apparent under physiological or pathological stress (5). Several published reports exist related to the potential physiological roles of these lipids in various tissue types, but few studies have investigated their

Fig. 7. Quantitative data from intestinal lipid analysis. Shown are data from 6 rats in each group from 2 independent experiments. Other lipids detected that did not show differences between the control and iron-deficient groups include arachidonic acid, linoleic and linolenic acids, 9HODE, 13HpODE, and 9HpODE. *P values (control vs. iron-deficient): 12-HETE, duodenum 0.04, jejunum 0.002; 13-HOTE, duodenum 0.007, jejunum 0.01; 13-HODE, jejunum 0.007. Error bar represents SD; statistics by t-test. Numbers are lipid concentrations (µM).
roles in intestinal physiology. It has been reported that Alox15 is induced at sites of active inflammatory bowel disease (39), suggesting a role in the inflammatory process. Another recent report suggested that Alox15 is involved in intestinal epithelial cell differentiation and apoptosis (27), implying a potential role in intestinal carcinogenesis. During iron deficiency, inflammation per se does not exist, so the more likely possibility is that Alox15 lipid products could alter differentiation of the intestinal mucosa. In fact, recent studies have demonstrated altered epithelial structure during iron deficiency (15, 40, 45), including increased villus height and width, increased crypt depth, and enterocyte migration rate. We confirmed these previous observations by performing extensive histopathological analysis of small intestinal sections harvested from groups of control and iron-deficient rats; results showed increased villus length, crypt width, mucosal thickness, and numbers of mitotic cells. Increased cell division in the intestinal crypts during iron deficiency is a novel observation that may be a compensatory mechanism to enhance iron uptake by increasing the number of absorptive enterocytes and increasing the rate of cellular differentiation. In fact, Smith et al. (40) recently reported that intestinal epithelial cells near the crypt-villus junction participate in iron absorption during iron deficiency, whereas only enterocytes further up the villus are involved in control animals. These physical alterations in the gut epithelium may represent an adaptive response to increase nutrient uptake in general and iron uptake specifically, since iron deficiency impairs growth and reduces energy levels, particularly in children. Another possibility is that activity of 12-lipoxygenases induces physical changes in the cell by peroxidizing membrane lipids and inducing a series of programmed structural changes (5). The net effect could again be related to increasing nutrient uptake.

In summary, we have progressed from observations made utilizing gene chips to metabolic changes in the intestine of a living mammal. Our genomewide profiling studies have identified a short list of candidate genes for further analysis; the Alox15 gene was one of the most interesting candidates. We demonstrated that Alox15 is strongly induced throughout the length of the mammalian intestine and we hypothesize that this induction results in the production of biologically active lipid mediators. The precise role of these lipids in intestinal physiology will be the subject of further studies. The roles of 12-lipoxygenases in common pathologies of the gastrointestinal tract, such as inflammatory bowel disease

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### Table 3. Histopathological analysis of proximal small intestine of experimental rats

<table>
<thead>
<tr>
<th></th>
<th>Villus Height*</th>
<th>Villus Width*</th>
<th>Crypt Depth*</th>
<th>Crypt Width*</th>
<th>Mucosal Thickness*</th>
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<td>Control</td>
<td>0.601±0.037</td>
<td>0.118±0.013</td>
<td>0.143±0.014</td>
<td>0.038±0.005</td>
<td>0.747±0.045</td>
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<tr>
<td>Low Fe</td>
<td>0.663±0.068</td>
<td>0.110±0.011</td>
<td>0.139±0.014</td>
<td>0.043±0.004</td>
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<td>P Value</td>
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<td>NS</td>
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<tr>
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<th>Mitosis‡</th>
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<tr>
<td>Control</td>
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<td>8.98±1.37</td>
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<tr>
<td>Low Fe</td>
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</tr>
<tr>
<td>P Value</td>
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<td>0.021</td>
</tr>
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</table>

*Measurements are in mm; average ± SD of 5 observations from 3 sections from each animal; †per 10 epithelial cells; average ± SD of 10 observations from 3 sections from each animal; ‡mitotic events per high power field; average ± SD of 10 observations of 3 sections from each animal. Sections from each animal were 1, 11, and 21 cm distal of the pyloric sphincter; n = 6 control rats and 7 iron-deficient rats for each parameter; NS, not significant (i.e., P > 0.05).
and colon cancer, raise intriguing possibilities for investigating the activity of Alox15 produced lipid mediators in additional models related to anemia of chronic disease.

GRANTS

These studies were supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants 1R21 DK068349 (J. F. Collins) and 1R01 DK074867 (J. F. Collins).

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