Iron Treatment Downregulates DMT1 and IREG1 mRNA Expression in Caco-2 Cells

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ABSTRACT Iron deficiency is the most common nutritional disorder worldwide, whereas pathologic elevations of body iron stores can occur under certain circumstances due to genetic abnormalities or in association with other diseases. The intestine is the exclusive locus of homeostatic regulation of body iron stores, which is accomplished by changes in iron absorption efficiency by largely unknown molecular mechanisms in response to alterations in body iron stores. Recently, a number of novel genes involved in iron metabolism, such as the iron uptake transporter DMT1/DCT1/Nramp2 and the iron export transporter IREG1/ferroportin1/MTP1, have been identified, providing important insights about molecular aspects of intestinal iron absorption and its regulation. The aim of this study was to investigate the effects of iron treatment on DMT1 and IREG1 mRNA expression in Caco-2 cells, a human intestinal cell line. Exposure of the cells to iron (200 μmol/L ferric nitrilotriacetic acid for 72 h) significantly decreased transferrin receptor mRNA (80%), DMT1 mRNA (57%) and IREG1 mRNA (52%). These observations are consistent with the notion of parallel regulation of these iron-responsive genes in vivo to protect the enterocyte from iron toxicity and mediate a decreased efficiency of intestinal iron absorption to prevent iron overload. J. Nutr. 132: 693–696, 2002.

KEY WORDS: iron absorption, ferroportin1, DCT1, Nramp2, iron responsive protein, MTP1

Iron deficiency is a public health problem of global importance. At the other end of the spectrum, iron overload from primary genetic disorders, such as hereditary hemochromatosis or other causes, can lead to considerable tissue damage and increase subsequent disease risk. Once dietary iron is absorbed, there is no regulatory mechanism for removing iron from the body. Thus, in the absence of blood loss, absorbed iron is largely retained in the body and can accumulate throughout life. The primary focal point of whole-body iron homeostasis is the control of intestinal iron absorption efficiency. In iron deficiency, a marked elevation of iron absorption occurs, whereas increases in body iron stores lead to lower absorption efficiency. This regulatory mechanism is highly efficient (1). Absorbed iron must transverse the brush border membrane of the enterocyte, navigate through the cytoplasm and finally exit the cell across the basolateral membrane. However, until recently, the molecular details of membrane iron transport in the gut have been largely unknown (2). In the past several years, a number of novel genes (3–8) have been identified that code for proteins that are important in intestinal iron absorption or its regulation. The influx of dietary nonheme iron from the intestinal lumen into the enterocyte is mediated by the specific brush border membrane iron transporter DMT1/DCT1/NRAMP2 (4,9). A candidate basolateral iron transporter, IREG1/ferroportin1/MTP1, was described recently (7,10,11). The mRNAs of both DMT1 (4) and IREG1 (7) have an iron response element (IRE) motif. In IREG1, the IRE motif is found in the 5’ untranslated region (UTR) of the mRNA, as occurs in ferritin mRNA and some other iron-responsive proteins (12). In the IRE-containing isoform of DMT1 (13), the IRE motif is found in the 3' UTR of the mRNA, as occurs in the transferrin receptor (TfR) mRNA. The differential positioning of these mRNA IRE motifs provides a possible mechanism for coordinating the translations of these proteins in a reciprocal manner in response to changes in cellular iron status through the singular activity of an iron responsive protein (IRP), as seen for example with ferritin and the TfR protein expression (14). This may also be the case for HFE and DMT1, which are coordinated in a reciprocal manner by cellular iron status in Caco-2 cells (13).

Caco-2 cells are a human colon adenocarcinoma cell line that spontaneously differentiate in culture and display a small intestine-like phenotype (16). Previous studies have shown that iron transport across monolayers of Caco-2 cells is regulated by iron treatment (17,18). In the present study, we have shown that iron treatment sufficient to change the expression of ferritin and TfR protein levels significantly decreases both DMT1 and IREG1 mRNA in Caco-2 cells.
from Sigma Chemical (St. Louis, MO). Glutamine, nonessential amino acids, pyruvate, penicillin G, streptomycin and gentamycin were from Gibco BRL (Gaithersburg, MD). Unless otherwise noted, all other reagents were purchased from Sigma Chemical or Fisher Scientific (Springfield, NJ).

**Cell culture and iron treatments.** Caco-2 cells (HTB 37) were obtained from American Type Culture Collection (Rockville, MD) and used between passages 35 and 50. Stock cultures were grown in Dulbecco's modified Eagle's medium containing 10% FBS, 25 mmol/L glucose, 2 mmol/L glutamine, 100 mmol/L nonessential amino acids, 1 mmol/L pyruvate, 50 µg/ml gentamycin, 100 µg/ml penicillin G and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2. For experiments, cells were either grown in 6-well plastic dishes seeded at 2 x 10^5 cells/well or on permeable membrane filter supports (Transwell, 0.4-µm pores; Corning, Cambridge, MA) seeded at 2.5 x 10^4 cells/well. Cells were used after 15 d in culture when cells had differentiated and demonstrated a small intestine-like phenotype (19,20). Two days before iron treatment was initiated, the FBS concentration was reduced from 10 to 0 and 2% in the medium added to the apical and basolateral side of the monolayer, respectively, or from 10 to 2% for cells grown on plastic, for reasons previously described (15). Cell monolayers grown on plastic were then treated on their apical side with various concentrations (50-800 µmol/L) of ferric nitroprussiate acid (Fe-NTA) plus 1 mmol/L ascorbic acid for 72 h, as indicated. Because previous studies have shown that iron treatment decreased transepithelial transport in Caco-2 cells were done in cells grown on permeable filter supports, we also wanted to evaluate whether a change in iron-responsive gene expression also occurs under these conditions. In these experiments, however, the treatment protocol was changed in several ways. The amount of iron used was reduced and the form of iron used in the treatment was changed (30 µmol/L deferin transferrin). In addition, the iron was presented to the cells for 72 h but exclusively from the basolateral side of the monolayer where iron uptake is mediated through the transferrin receptor.

**Reverse transcriptase-polymerase chain reaction.** Total RNA was isolated from Caco-2 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH). The first strand of cDNA was obtained by standard reverse transcriptase (RT) reaction. The cDNA was used as a template for polymerase chain reaction (PCR) amplification with Taq polymerase (Perkin-Elmer, Norwalk, CT). Primer sets were as follows: DMT1 (accession number XM 051168.1), forward primer, position 786–805 (5′-AAGCGAACGCCAGAGC-CAC-GTA-3′) and reverse primer, position 1157–1176 (5′-CCCCCCTTGTAGATTGCAC-3′), β-actin (accession number XM 037239), forward primer, position 861–882 (5′-CCTCTCTTGGGCAATGAGCTTCG-3′) and reverse primer, position 1042–1062 (5′-GGAGAACATGATCTGTTCCC-3′), IREG1 (accession number AF231121), forward primer, position 245–267 (5′-TCAGC-GAGAAGCAGCAAGCAGCAG-3′) and reverse primer, position 669–691 (5′-GGAACCCATCCATCGTGACT-3′). PCR was performed within the linear range of amplification. The density of PCR products visualized on ethidium bromide-stained gels was determined by using quantitation software (Quantify One, Bio-Rad Laboratories, Hercules CA) after digital capture of the gel image on a Gel Doc 2000 gel documentation system (Bio-Rad). Data for TFR, IREG1 and DMT1 mRNA expression were normalized to the levels of β-actin mRNA, a constitutively expressed gene.

**Transferrin receptor Western blotting.** Cells were lysed in buffer containing 50 mmol/L Tris-HCL, 300 mmol/L KCl, 1.5 mmol/L EDTA, pH 7.4, 0.3 mmol/L PMSE, 1 mg/L pepstatin A, 20 mg/L soybean trypsin inhibitor, 500 kallikrein inhibitor units (KIU)/mL aprotinin and 5 mmol/L dithiothreitol. Aliquots of proteins were heated to 100°C for 5 min in Laemmli SDS buffer. These samples were then separated by SDS-polyacrylamide gel electrophoresis using a 10% Tris-glycine gel and electroblotted onto nitrocellulose membrane (Amersham, Piscataway, NJ). The blots were then incubated with mouse anti-human TIR antibody (Zymed, San Francisco, CA) followed by incubation with horseradish peroxidase-linked antimouse immunoglobulin G antibody. Transferrin receptor was detected using an ECL chemiluminescent assay (Amersham) after exposing the blot to film and measured by densitometry.

**Ferritin ELISA.** Cells were grown in plastic cell culture dishes as described above. Two days before initiation of iron treatment, the level of FBS in the cell culture medium was reduced from 10 to 2%. An immunoradiometric assay (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) was used to measure Caco-2 cell ferritin content, as described by Glahn et al. (21).

**Statistics.** Data are presented as means ± SEM. Significant treatment differences were determined by paired t test, or linear regression analysis, as indicated, using SYSTAT 9 for Windows statistical software (SPSS, Chicago IL). Differences with P < 0.05 were considered significant.

## RESULTS

**Iron treatment of Caco-2 cells alters cellular iron status.** Expected changes in cellular iron status in Caco-2 cells grown on plastic treatment after addition of 200 µmol/L Fe-NTA for 72 h were first confirmed by measuring changes in TFR and ferritin protein. Iron treatment decreased the level of TFR protein (1264 ± 145 vs. 953 ± 175 density units, P < 0.03, paired t test). Figure 1 illustrates the iron-dependent changes in TFR protein level in four separate studies. Similarly, as expected, iron treatment increased the level of ferritin protein from very low levels in control cells not receiving additional iron to markedly elevated levels in iron-treated cells (6.7 ± 0.06 to 519 ± 358 ng cell ferritin/mg cell protein). These findings demonstrate that this level of iron treatment in Caco-2 cells was sufficient to predict cellular iron status and plausibly change the expression of these two well-known iron-dependent proteins in a reciprocal manner.

**Iron treatment decreases both DMT1 and IREG1 mRNA.** Iron treatment (200 µmol/L Fe-NTA for 72 h; n = 6) of Caco-2 cells grown on plastic dishes significantly decreased mRNA levels of TFR (80% decrease; P < 0.0001), DMT1 (57% decrease; P < 0.025) and IREG1 (52% decrease; P < 0.001; Fig. 2). Also, in a dose-response experiment, there was a linear dose-dependent reduction in both TFR mRNA (r = −0.89, P < 0.001) and IREG1 mRNA (r = −0.83, P < 0.001). In studies done in Caco-2 cells grown on permeable filter supports, we also noted a decrease in expression of these iron-responsive genes, although overall, the relative changes were less marked. The mean expression level of TFR mRNA was significantly (P < 0.005) decreased by 31% in the iron-treated group compared with control (1.00 ± 0.08 vs. 0.69 ± 0.15, expressed in arbitrary relative units). Similarly, IREG1 mRNA was significantly (P < 0.005) decreased by 19% in the iron-treated group (1.08 ± 0.06 to 0.81 ± 0.07).

## DISCUSSION

We found that treatment of the Caco-2 human intestinal cell line with iron sufficient to change TFR and ferritin protein

**Figure 1** Iron treatment decreases transferrin receptor (TFR) protein in Caco-2 cells. Fifteen-day cultures of Caco-2 cells grown on plastic dishes were treated with growth medium containing 2% fetal bovine serum and either 0 (−Fe) or 200 µmol/L Fe-NTA (+Fe) for 72 h. Whole-cell protein homogenates were subjected to Western analysis for the detection of TFR protein. This figure shows the iron-dependent reduction in the expression of TFR protein found in four different experiments. Mean TFR protein was significantly (P < 0.03, paired t test) decreased by iron treatment (see text).
levels was associated with a reduction in DMT1 and IREG1 mRNA.

The iron-dependent change of DMT1 mRNA in Caco-2 cells was significantly lower (TfR mRNA $P < 0.0001$; DMT1 mRNA $P < 0.025$; IREG1 mRNA $P < 0.01$) in the iron-treated group. Our observation of a decrease in the level of TfR mRNA and DMT1 mRNA after iron treatment is consistent with the current view of the mechanism of IRE-mediated downregulation of mRNA. In these cases, mRNA stability is affected through the iron-dependent dissociation of an iron regulatory protein from the IRE motif(s) found in the 3' UTR of both DMT1 and TfR mRNA (14). Reduced TfR levels, in the face of raised intracellular iron concentration in the enterocyte after iron treatment, would normally serve to protect the enterocyte from iron toxicity stemming from an unabated uptake of iron across the basolateral membrane from circulating holo-transferrin. Reduced DMT1 mRNA in the enterocyte after iron treatment would lead to a reduced synthesis of the DMT1 iron transporter and reduced dietary iron uptake from the gut, which would be consistent with the well-known homeostatic decrease in intestinal iron absorption efficiency accompanying high body iron stores. This feedback loop is physiologically important because an inability to properly downregulate DMT1 and iron uptake into the enterocyte could result in inappropriately high rates of intestinal iron absorption, which could eventually cause iron overload because the body avidly retains absorbed iron. The fact that iron overload does not commonly occur in most people in the face of a chronic surfeit of dietary iron (22) supports the idea that this intestinal regulatory loop is highly effective at blocking excess iron absorption. On the other hand, certain genetic mutations may cause dysregulation of this important regulatory mechanism, resulting in high intestinal DMT1 expression and iron absorption despite excessive accumulation of body iron stores and may be important in the pathogenesis of hereditary hemochromatosis (23,24).

From a physiologic perspective, a coordinated regulation of iron influx and efflux rates in the enterocyte is likely to be of importance in maintaining iron homeostasis. In our study, we found that iron treatment of Caco-2 cells reduced the IREG1 mRNA for the intestinal basolateral membrane iron transporter (Fig. 2). To our knowledge, this is the first report of direct iron regulation of IREG1 in an intestinal cell line. Due to the recent identification of this novel gene, there is little information available about factors involved in the regulation of the intestinal IREG1/ferroportin1 basolateral iron transporter in the enterocyte or other cell types (7,25). However, the report of the presence of an IRE stem-loop motif in the 5' UTR of the IREG1 mRNA (7) suggests that IREG1 protein levels should be increased, not decreased, by an elevation of cellular iron status. This would follow the pattern of protein expression that we noted for ferritin, another iron-responsive protein with a 5' UTR mRNA IRE regulatory motif. In the case of ferritin, this increase in protein level occurs through an IRP-dependent mechanism, allowing increased translation of existing ferritin mRNA when cellular iron increases (14). However, we observed an iron-dependent reduction in IREG1 mRNA, which would seem to be at odds with a predicted iron-dependent increase in IREG1 protein under these conditions. Thus, it will be particularly important in future studies to measure the response of IREG1 protein levels to iron treatment. Unfortunately, we have been unsuccessful in a limited number of attempts to clearly detect IREG1 protein by Western blot in Caco-2 cells (data not shown). Alternatively, it is possible that a different splice variant of IREG1 mRNA that does not contain a 5' UTR IRE may occur in Caco-2 cells. The mechanism responsible for the lower IREG1 mRNA after iron treatment is unknown at the present time. This observation could reflect the outcome of various possibilities, including a direct effect of cellular iron status on IREG1 transcriptional activity, or an iron-dependent decrease in IREG1 mRNA stability, but will require further study. However, the iron responsiveness of IREG1 expression in Caco-2 cells suggests that they may be a useful cell culture model with which to further investigations of the molecular aspects of IREG1 involvement in iron transport in the enterocyte and factors that control its expression.

It is worth noting that a recent report (25) of ferroportin1/IREG1 mRNA and protein expression in duodenal biopsies of normal subjects and patients with hereditary hemochromatosis found a negative relation of both DMT1 and ferroportin1/IREG1 mRNA and protein with iron status in normal subjects, and inappropriately high levels of expression of these proteins in patients with hereditary hemochromatosis. These findings are consistent with our present finding in the Caco-2 human intestinal cell line of apparent parallel regulation of both DMT1 and IREG1. Collectively, these observations agree with the notion that homeostatic adjustments of intestinal iron absorption are achieved by a coordinated iron-dependent regulation of both the import and export iron transporters in the enterocyte.

Finally, in consideration of our apparent paradoxical findings, we would like to note that the control of IREG1 expression by iron in different cell types or organs might be complex. We speculate on functional grounds that the expected iron-
dependent regulation of IREG1 mRNA expression through the 5’ UTR IRE mechanism may be silenced in the enterocyte, and perhaps in other organs such as the liver, because these structures play vital regulatory roles in iron absorption and iron storage. In these organs, an iron-dependent increase in IREG1 expression and subsequently increased iron export under conditions in which cellular iron levels are high could be detrimental to the safety and overall iron economy in the body. Instead, in these organs, when cellular iron concentrations are high, it may trigger a compensatory, and apparently paradoxical, downregulation of IREG1, which would lead to a decrease in iron absorption and a condition favoring retention of iron in the liver, thereby providing protection against iron overload and iron-dependent tissue damage. In contrast, in other cell types (e.g., macrophages), the expected IRF-dependent increase in IREG1 expression might occur as an important way to facilitate iron efflux and the release of scavenged iron for hemoglobin regeneration, or as a general autoregulatory mechanism to simply protect cells from excessive iron accumulation and possible iron-induced oxidant damage. Additional levels of control of cellular iron transport may also be operative, at least in the intestine, through the action of other factors, such as the basolateral membrane ferrooxidase hephaestin (8), which can modulate iron efflux, and the iron-dependent brush border membrane ferric reductase (6), which may influence iron uptake, although this is uncertain in Caco-2 cells (26).

LITERATURE CITED