Iron Loading Increases Ferroportin Heterogeneous Nuclear RNA and mRNA Levels in Murine J774 Macrophages\textsuperscript{1,2}

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Abstract
The transmembrane protein ferroportin is highly expressed in tissue macrophages, where it mediates iron export into the bloodstream. Although ferroportin expression can be controlled post-transcriptionally through a 5’ iron-responsive element in its mRNA, various studies have documented increased ferroportin mRNA levels in response to iron, suggesting transcriptional regulation. We studied the effect of iron loading on levels of macrophage ferroportin mRNA, as well as heterogeneous nuclear RNA (hnRNA), the immediate product of ferroportin gene transcription. J774 cells, a mouse macrophage cell line, were incubated for 0, 3, 6, 9, 12, and 24 h in medium supplemented or not with 200 \textmu mol/L iron. Quantitative RT-PCR was used to measure steady-state levels of ferroportin mRNA and hnRNA. Ferroportin mRNA levels increased by 12 h after iron treatment, reaching 6 times the control levels after 24 h. Changes in ferroportin mRNA levels were paralleled by similar changes in the levels of ferroportin hnRNA. Time course studies of ferroportin mRNA and hnRNA abundance after incubating cells with the transcriptional inhibitor actinomycin D revealed that ferroportin mRNA has a half-life of \textasciitilde 4 h and that iron loading does not stabilize ferroportin mRNA or hnRNA. Collectively, these data are consistent with the hypothesis that iron increases macrophage ferroportin mRNA levels by inducing transcription of the ferroportin gene. J. Nutr. 139: 434–438, 2009.

Introduction
Resident macrophages of the spleen, liver, and bone marrow play a central role in whole-body iron balance by recycling systemic iron (1). These macrophages, collectively known as the reticuloendothelial (RE)\textsuperscript{3} system, recycle iron by ingesting old or damaged erythrocytes and catabolizing the hemoglobin to liberate iron. The liberated metal is either stored in the cell or exported into the bloodstream. Each day in the average adult human, RE macrophages recycle 20–25 mg of iron, an amount that is 10–20 times more than the intestine absorbs from the diet.

Export of iron from the macrophage is mediated by ferroportin (2), a transmembrane iron transport protein located on the cell surface (3). In mice engineered to lack ferroportin, iron accumulates in hepatic and splenic macrophages, indicating that ferroportin is the primary, if not only, iron export protein in RE cells (4). The critical role of ferroportin in macrophage iron efflux is further highlighted by an ever-growing number of clinical reports of ferroportin mutations associated with pathologic macrophage iron accumulation (5). Some of these mutations lead to a loss of ferroportin-mediated iron export, as indicated by cell culture studies (6,7).

A key advance in our understanding of ferroportin in iron metabolism came from the discovery of hepcidin, a peptide hormone that regulates systemic iron homeostasis (8). Produced and secreted into the circulation by the liver, hepcidin binds to ferroportin at the cell surface and causes its internalization and degradation (2,9). This post-translational degradation of ferroportin by systemic hepcidin therefore determines the amount of iron released into the plasma. In the absence of hepcidin (10), cellular mechanisms primarily control ferroportin expression. One of these mechanisms includes post-transcriptional regulation through iron-regulatory proteins (IRP), which bind to mRNA stem loop structures called iron-responsive elements (IRE). Ferroportin mRNA contains an IRE in its 5’ untranslated region similar to the mRNAs encoding ferritin L and ferritin H, 2 subunits of the iron storage protein ferritin (11). The post-transcriptional regulation of ferritin expression by IRE-IRP interactions is well characterized (12). Under low iron conditions, IRP bind to the 5’ IRE in ferritin mRNA and block its translation. When iron levels are high, IRP are either degraded or fail to bind IRE with high affinity, allowing for translation. The 5’ IRE of ferroportin shows a similar iron-dependent regulation of translation in the mouse macrophage cell line,
RAW 264.7 (13). In vivo, iron levels in RE macrophages are normally high, because these cells regularly ingest senescent erythrocytes. Under these high iron conditions, IRP degrade and no longer exert post-transcriptional control through IRE interactions. A more important determinant of macrophage ferroportin expression in this state, therefore, would be the pool of ferroportin mRNA available for translation.

In contrast to ferritin, the amount of macrophage ferroportin mRNA varies substantially with cellular iron status, increasing in iron loading and decreasing in iron deficiency (14–17). The variations in ferroportin mRNA levels may reflect differences in transcription rate or mRNA stability. Studies using the transcriptional inhibitor actinomycin D provide support that macrophage ferroportin is regulated transcriptionally by iron (15,16). In the present study, we examined the relationship between levels of ferroportin mRNA and pre-mRNA [heterogeneous nuclear RNA (hnRNA)]. The levels of specific hnRNA, which represent precursors of mature mRNAs, are increasingly being used as an indicator of transcriptional activity (18).

Materials and Methods

Cell culture and treatments. J774 cells, a murine macrophage cell line, were cultured in a minimum essential medium (Mediatech) supplemented with 10% fetal bovine serum (Cambrex), penicillin, and streptomycin and incubated at 37°C in 5% CO2. Cells were incubated in medium supplemented or not with 200 μmol/L ferric nitrilotriacetic acid (Fe-NTA) for 0, 3, 6, 12, and 24 h. Fe-NTA (molar ratio of 1:4) was prepared as a 20-mmol/L stock from NTA (Sigma-Aldrich) and ferric chloride hexahydrate. Transcription was inhibited by incubating cells with actinomycin D (1 μg/mL Sigma-Aldrich) and ferric chloride hexahydrate. Transcription was inhibited by incubating cells with actinomycin D (1 μg/mL Sigma-Aldrich) for up to 8 h in the presence or absence of 200 μmol/L Fe-NTA.

Measurement of ferroportin mRNA and hnRNA abundance. Relative abundances of specific RNA were determined by using quantitative RT-PCR (qRT-PCR). Briefly, total cellular RNA was isolated from J774 cells by using RNA Bee (Tel-Test). Isolated RNA was treated with DNase 1 (Turbo DNA-free kit, Ambion) to remove any contaminating genomic DNA. First-strand cDNA was synthesized from the isolated RNA (1 μg) using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBRGreen Supermix (Bio-Rad) and isolated RNA (1 μg) using the iScript cDNA synthesis kit (Bio-Rad) and an Applied Biosystems 7300 real-time PCR system. Quantitation of mRNA was determined by comparison to standard curves generated by 4 10-fold dilutions of standard cDNA. To investigate changes in transcriptional intermediates, we used qRT-PCR to measure steady-state transcript levels by message stabilization. To examine variations in ferroportin hnRNA levels in the macrophage.

Iron loading increases ferroportin mRNA and hnRNA abundance. Relative abundances of specific RNA were determined by using quantitative RT-PCR (qRT-PCR). Briefly, total cellular RNA was isolated from J774 cells by using RNA Bee (Tel-Test). Isolated RNA was treated with DNase 1 (Turbo DNA-free kit, Ambion) to remove any contaminating genomic DNA. First-strand cDNA was synthesized from the isolated RNA (1 μg) using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using iQ SYBRGreen Supermix (Bio-Rad) and an Applied Biosystems 7300 real-time PCR system. Quantitation of mRNA was determined by comparison to standard curves generated by 4 10-fold dilutions of standard cDNA. To investigate changes in transcriptional intermediates, we used qRT-PCR to measure steady-state transcript levels by message stabilization. To examine variations in ferroportin hnRNA levels in the macrophage.

Results

Iron loading increases ferroportin mRNA and hnRNA levels in the macrophage. To assess the effect of iron on ferroportin pre-mRNA and mRNA abundance, we used qRT-PCR to measure steady-state levels of ferroportin mRNA and hnRNA in J774 mouse macrophages treated with 200 μmol/L Fe-NTA for 0, 3, 6, 9, 12, and 24 h. This amount of Fe-NTA maximally induces ferroportin mRNA expression in J774 cells, as determined by Northern blot analysis (16). In response to iron treatment, ferroportin mRNA abundance increased progressively over time. By 12 h, ferroportin mRNA levels increased 3-fold over controls (P < 0.01), reaching 5-fold higher levels (P < 0.001) after 24 h (Fig. 2A). A similar temporal response and magnitude of increase was observed in ferroportin hnRNA levels (Fig. 2B).

Effect of iron loading on ferroportin mRNA and hnRNA stability. The parallel increases in ferroportin hnRNA and mRNA levels suggest that iron increases ferroportin gene transcription. Nonetheless, it is also possible that iron increases steady-state transcript levels by message stabilization. To examine this possibility, J774 cells were incubated with or without 200 μmol/L Fe-NTA for 12 h, followed by treatment with the transcriptional inhibitor actinomycin D for 0, 2, 4, 6, and 8 h. Treatment of iron-loaded cells with actinomycin D decreased ferroportin mRNA levels over time compared with cells not treated with actinomycin D (Fig. 3A). In these same cells, actinomycin D treatment decreased ferroportin hnRNA levels more potently (Fig. 3B). After 2 h of treatment, ferroportin mRNA levels decreased by >80% (P < 0.001). Decreases in macrophage ferroportin mRNA and hnRNA levels after acti-

Iron increases ferroportin hnRNA and mRNA

**FIGURE 1** Diagram of the mouse ferroportin gene, hnRNA, and mRNA. The mouse ferroportin gene is located in a 17,524-bp region (NC_000067) on the minus strand of chromosome 1, positions 45964915–45982439. Levels of ferroportin hnRNA were determined by using qRT-PCR and primers targeting a region that spans the 3rd intron-exon junction. Ferroportin mRNA (NM_016917) abundance was determined by using qRT-PCR and primers targeting a region in exon 6.
Effect of iron loading on ferritin L mRNA levels. Treatment of J774 cells with 200 μmol/L Fe-NTA increased ferritin L mRNA levels by iron is the reason for the differential responses of ferritin L and ferroportin mRNA to iron loading may relate to their relative abundances. By our qRT-PCR analyses of J774 macrophages, we estimate that basal ferritin L mRNA levels are at least 3 orders of magnitude greater than ferroportin mRNA levels. This estimate is consistent with a serial analysis of gene expression in human monocyte-derived macrophages, which identified ferritin L as the single most abundantly expressed mRNA of 35,000 different transcripts (26). With such a large supply of pre-existing ferritin L mRNAs, the production of sufficient ferritin L protein in response to iron loading can occur without requiring additional transcription. Ferroportin message, in contrast, is not abundant at the single-copy level and mRNA stability (23) rather than an increase in transcription is required to increase ferroportin pre-mRNA. Moreover, these results provide evidence that transcription is required to increase ferroportin hnRNA and mRNA levels in response to iron. It therefore appears that iron increases ferroportin expression primarily by increasing transcription.

Although ferroportin mRNA levels increased markedly 12 h after iron treatment, ferritin L mRNA levels did not change. Similar responses for ferroportin and ferritin L mRNA have been reported in studies of bone marrow-derived macrophages loaded with erythrocyte iron (14). It is important to note that, despite the lack of change in ferritin L mRNA abundance in response to iron, macrophage ferritin L protein levels increase markedly (14). This demonstrates that macrophage ferritin L expression is regulated mostly in a post-transcriptional manner by iron. We did, however, observe a 2-fold increase in ferritin L mRNA levels after loading cells with iron for 24 h. Because we were unable to measure ferritin L hnRNA, it seems that the increase in ferritin L mRNA levels in response to iron results from an increase in mRNA stability (23) rather than an increase in transcription (24,25).

The differential responses of ferritin L and ferroportin mRNA to iron loading may relate to their relative abundances. By our qRT-PCR analyses of J774 macrophages, we estimate that basal ferritin L mRNA levels are at least 3 orders of magnitude greater than ferroportin mRNA levels. This estimate is consistent with a serial analysis of gene expression in human monocyte-derived macrophages, which identified ferritin L as the single most abundantly expressed mRNA of 35,000 different transcripts (26). With such a large supply of pre-existing ferritin L mRNAs, the production of sufficient ferritin L protein in response to iron loading can occur without requiring additional transcription. Ferroportin message, in contrast, is not abundant at the single-copy level and mRNA stability (23) rather than an increase in transcription is required to increase ferroportin pre-mRNA. Moreover, these results provide evidence that transcription is required to increase ferroportin hnRNA and mRNA levels in response to iron. It therefore appears that iron increases ferroportin expression primarily by increasing transcription.

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The present study suggests that transcription is an important means of modulating ferroportin expression. If so, ferroportin transcription would be high in the usually iron-rich RE macrophage, whereas post-transcriptional regulation (i.e. suppression of translation) through IRE/IRP interactions would be low. Indeed, ferroportin transcription would be a primary determinant of macrophage ferroportin expression when hepcidin levels are low, such as in anemia (28), hypoxia (28), states of enhanced erythropoiesis (29), HFE-related hemochromatosis (30), and juvenile hemochromatosis (31).

Although iron loading consistently increases ferroportin mRNA levels in macrophages in vivo (17,32) and in cultured macrophages (2,14–16), it has the opposite or no effect in the duodenum (32), isolated enterocytes (33,34), or cultured intestinal cells (34–36). Moreover, iron deficiency has been shown to decrease ferroportin mRNA levels in macrophages (15,16) but increase ferroportin mRNA levels in intestinal cells (11,32–34). In one of these studies (34), the increase in ferroportin mRNA abundance was shown to involve transcription, as demonstrated by nuclear run-off analysis. Studies of isolated enterocytes from mice with nutritional and genetic iron deficiency indicate that intestinal ferroportin mRNA (and protein) levels respond to systemic signals rather than local iron concentration (33). We conclude from the present study that macrophage ferroportin expression responds to local iron concentrations (likely via transcription) in addition to systemic signals such as hepcidin.

Future studies of cell type-specific regulation of ferroportin will be needed to identify the cis and trans regulatory elements involved in the iron-dependent regulation of ferroportin transcription.

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Literature Cited


