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Iron responses in hepatic, intestinal and macrophage/monocyte cell lines under different culture conditions

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Abstract

Iron homeostasis is mainly controlled by the liver-produced hepcidin peptide, which induces the degradation of the ferroportin iron exporter and thus regulates serum iron level. Hepcidin transcription is clearly up-regulated by the pro-inflammatory cytokine IL-6 and down-regulated, in the case of iron depletion, at least via HIF transcription factors. In addition, *in vivo* iron overload up-regulates hepcidin, but this cannot be reproduced in cell culture or isolated hepatocytes. Here, we investigated the steady state mRNA levels of a series of genes involved in iron metabolism in hepatic HepG2, intestinal Caco-2, and monocyte/macrophage THP-1 cell lines under different iron and culture conditions. Our results showed that iron-saturated transferrin up-regulated hepcidin mRNA synthesis from HepG2 via cross-talk with macrophages or enterocyte cytokine-producing cells, whereas non-transferrin-bound iron down-regulated hepcidin, likely due to missing TfR-iron-transferrin uptake.

Keywords: Iron responses; Holo-transferrin; NTBI; Quantitative RT-PCR; Cell culture conditions

Introduction

In humans, iron homeostasis is regulated at organismal and intracellular levels by two different mechanisms. In the first mechanism, serum iron depends on the hepcidin peptide expressed in the liver, which binds to the ferroportin iron exporter and induces its internalization and degradation [1–3]. Thus, this molecular pathway mainly down-regulates iron release from enterocytes and macrophages, which are involved in iron absorption from food and erythrocyte–iron recycling. *In vivo*, hepcidin mRNA and protein expression are enhanced by iron loading and inflammation, and are decreased by erythropoiesis and hypoxia [1,2,4]. The proinflammatory cytokines IL-6 and IL-1 are known to up-regulate mRNA hepcidin expression and may contribute to the anemia of chronic inflammation [1,4–7]. The hypoxia-inducible transcription factors (HIFs) may be one mechanism of hepcidin inhibition during iron depletion as they were recently shown to coordinately down-regulate hepcidin and up-regulate erythropoietin and ferroportin through the iron-dependent propyl hydroxylases (PHDs) pathway [8]. Moreover, according to this study, HIFs prevent the proinflammatory cytokine IL-6- and IL-1-mediated stimulation of hepcidin expression.

The second mechanism is post-transcriptional, based on the IRE/IRP iron-dependent system, and coordinates the synthesis of certain proteins involved in intracellular iron metabolism [9,10]. A high level of intracellular labile iron prevents the iron regulatory proteins IRP1 and IRP2 from binding to the RNA iron-responsive element (IRE) found in the 5′ untranslated region of L- and H-chain ferritins, and in the 3′ untranslated region of *TFRC* mRNAs; the former enhances protein synthesis, whereas the latter destabilizes mRNA and induces down-regulation. In addition, the intestinal form of the transmembrane divalent-metal transporter 1 (DMT1) mRNA (*SLC11A2*) carries a 3′-UTR IRE that blocks additional iron absorption after accumulation of intracellular iron. In other cells, the DMT1 protein required for the recycling of iron from the TfR1–Tf–Fe endocytic cycle is encoded by a non-IRE mRNA [11,12].

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In humans, the control of hepcidin expression, which regulates dietary iron absorption by duodenal enterocytes, plays a critical role as there is no pathway of excretion. Hepcidin defects cause a recessive juvenile severe iron overload due to the missing down-regulation of intestinal iron absorption [13], and some mutant ferroportin forms are associated with an autosomal dominant disorder due to abnormal iron distribution [14]. Some other proteins involved in the control of iron levels are also implicated in iron overload diseases. Hence, the most common form of iron overload disease is the autosomal recessive *HFE*-linked form of hemochromatosis [15]. HFE interacts with TfR1 at the cell surface competitively with iron-bound transferrin [16,17]. Transferrin receptor 2 (TfR2), a homolog of TfR1 mainly expressed in hepatocytes, competes with TfR1 for binding to HFE [18], and TfR2 protein stability is regulated by diferric transferrin [19–21]. The findings that TfR2-KO mice develop iron overload and are unable to upregulate hepcidin expression, and that the haemoglobin-deficient mouse strain, *hbd,* shows increased transferrin saturation and levels of hepatic hepcidin all suggest that TfR2 is a sensor of diferric transferrin [22,23]. In addition, *TFR2* and hemojuvelin *(HJV*), which positively regulate hepcidin mRNA expression, have been shown to be involved in rare recessive adult and juvenile forms of hemochromatosis, respectively [24–26]. Both *HFE*- and *TFR2*-associated hemochromatosis patients, as well as *Hfe*−/− mice and TfR2-KO mice, exhibit high transferrin saturation and inappropriately low hepcidin expression, despite high liver iron contents [27,28]. HFE and TfR2 are molecular components necessary for maintenance of a normal iron balance; deficiencies in both result in similar symptoms [15,24]. These observations indicate that hepcidin is a common factor in the iron overload process of genetic hemochromatosis and that HFE, TfR2 and HJV act as upstream regulators of hepcidin expression. Obviously, *in vivo*, hepcidin expression is increased in experimentally iron-overloaded mice. However, in isolated hepatocytes or hepatic cell lines exposed to iron, hepcidin expression is decreased rather than increased; indeed isolated hepatocytes require serum from iron-overloaded individuals or activated monocytes to maintain hepcidin synthesis. [1,2,4,6]. These observations suggest an indirect mechanism for the up-regulation of hepatic hepcidin expression by iron. To gain more insight into the iron signaling mechanism in hepcidin regulation, we investigated steady state mRNA levels of iron metabolism genes as a function of iron status in independently cultured hepatic, intestinal and monocyte/macrophage cell lines, as well as in hepatic cells co-cultured with intestinal cells or cultured with macrophage-conditioned medium.

Materials and methods

Cell culture

Human HepG2 (hepatocellular carcinoma HB-8065), Caco-2 (intestinal adenocarcinoma HTB-29), and THP-1 (acute monocytic leukemia TIB-202) cell lines were obtained from the American Type Culture Collection. HepG2 and Caco-2 were maintained in Eagle's Minimum Essential Medium (ATCC), supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. These cell lines display some characteristics of the parent organ; in particular, Caco-2 cells differentiate after confluence and have a small intestine-like phenotype, and THP-1 monocytes differentiate into macrophages when exposed to phorbol 12-myristate 13-acetate (PMA). HepG2 cells were grown on 35-mm culture dishes seeded at 3×10^5 cells per well. Caco-2 were grown on 24-mm permeable polycarbonate membrane filter supports (Transwell, 0.4 μm pores, Corning, Cambridge, USA), and the formation of a complete cell monolayer was checked by measuring the transepithelial electrical resistance (TEER) with an epithelial voltohmmeter (EVOM™, World Precision Instruments, USA). At confluence, the normal TEER values ranged from 400 to 450 Ω/cm^2. Intestinal cell differentiation was checked by RNA villin amplification using primers sense 5′-ACCATCTTCTGCTGGGCA-3′ and antisense 5′-GAGCCATGCGCTGAA-3′. The analysis of villin mRNA levels along with cell growth on filter supports indicated that most of the Caco-2 cells were differentiated after TEER stabilization. Thus, all mRNA analyses in Caco-2 were conducted 48 h after TEER normalization and the lack of change in values at the time of treatment indicates that the integrity of the cell monolayer was unaffected. For compartmentalized HepG2 and Caco-2 cell co-culture experiments, membrane filters with confluent Caco-2 were transferred to culture dishes containing 80% confluent HepG2 cells. The medium was replaced every two days.

THP-1 cells were grown in RPMI-1640, supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Differentiation of THP-1 monocytes into macrophages was performed as described by Whatling et al. [29]. Briefly, cells plated at 4×10^5 cells/mL in 60-mm tissue culture plates containing 2 mL medium were stimulated with 50 ng/mL of PMA for 24 h. After removal of the medium, the cells were washed twice with PBS prior to the addition of fresh medium. After 24 h, the conditioned medium obtained from THP-1 cells prepared under these conditions was added to newly plated THP-1 monocytes further incubated for 24 h to allow cell differentiation in a manner precluding direct effects of PMA. Cell concentration and percentage of viable cells were determined by the trypan blue cell exclusion method.

Cell treatments

Cells were subjected to 100 μM ferric nitriloacetic acid (Fe-NTA), or to 4.5 g/L of iron-saturated human diferric transferrin (holo-TF). For iron treatment of HepG2 and Caco-2 cell monolayers, the medium corresponding to the basolateral side of Caco-2 was supplemented with Fe-NTA or holo-TF for 48 h before RNA extraction. For iron treatment of THP-1-differentiated cells, 24 h after the addition the differentiating medium, the medium was removed, cells were washed twice with 2 mL PBS and fresh medium containing Fe-NTA or holo-TF was added. Then, the 24 h iron-conditioned medium was added to HepG2 cells before incubation for 24 h and subsequent RNA extraction. Control experiments were carried out with apotransferrin and NTA alone. Other treatments of HepG2 cells were performed with 10 μg/mL of human IL-6 (R&D Systems Inc. Minneapolis, USA) or 100 μM desferrioxamine mesylate