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The iron regulatory hormone hepcidin inhibits expression of iron release as well as iron uptake proteins in J774 cells☆,☆☆

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Abstract

The mechanism by which hepcidin controls cellular iron release protein ferroportin 1 (Fpn1) in macrophages has been well established. However, little is known about the effects of hepcidin on cellular iron uptake proteins. Here, we demonstrated for the first time that hepcidin can significantly inhibit the expression of transferrin receptor 1 (TfR1) and divalent metal transporter 1 in addition to Fpn1, and therefore reduce transferrin-bound iron and non-transferrin-bound iron uptake and also iron release in J774 macrophages. Analysis of mechanisms using the iron-depleted cells showed that hepcidin has a direct inhibitory effect on all iron transport proteins we examined. Further studies demonstrated that the down-regulation of TfR1 induced by hepcidin is associated with cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA), probably being mediated by the cAMP–PKA pathway in J774 macrophages.

Keywords: Transferrin-bound iron (Tf-Fe) and non-transferrin-bound iron (NTBI); Transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1); Hepcidin peptide; cAMP and PKA

1. Introduction

Hepcidin is mainly synthesized by the liver [1–4]. Mice that fail to express hepcidin have elevated body iron stores, presumably due to hyperabsorption, associated with a decreased iron in tissue macrophages [5–7]. The synthesis of hepcidin is homeostatically increased by iron loading [8]. Hepcidin is also elevated during infections and inflammation [9], causing a decrease in serum iron levels and contributing to the development of anemia of inflammation [2]. Accumulated data have indicated that hepcidin is the principal regulator in the maintenance of systemic iron homeostasis [10,11]. Hepcidin controls plasma iron concentration and tissue iron distribution by inhibiting iron absorption in intestine, iron recycling by macrophages and iron mobilization from hepatic stores [10,11]. It has also been confirmed that the inhibitory role of hepcidin is initiated by a direct binding of this peptide with ferroportin 1 (Fpn1), an iron exporter present on the surface of absorptive enterocytes, macrophages, hepatocytes and placental cells [12–14]. Fpn1 is then internalized and degraded, leading to a decreased export of cellular iron [15].

However, little is known about the effects of hepcidin on cellular iron uptake proteins in macrophages. The results on the expression of transferrin receptor 1 (TfR1) in macrophages that received inflammatory stimuli are controversial. In addition, it is completely unknown whether hepcidin has a direct effect on the expression of iron uptake proteins TfR1 and/or divalent metal transporter 1 (DMT1) in the cells. In this experiment, we investigated the effects of hepcidin on iron uptake and release and expression of TfR1, DMT1 and Fpn1 in J774 macrophages.

2. Materials and methods

2.1. J774 rat macrophage cells

J774 rat macrophage cells (ATCC TIB-67) were grown at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 U/ml penicillin and 10 mg/ml streptomycin. The culture medium was changed every 2 days. The Animal Ethics Committees of Chinese University of Hong Kong and Fudan University approved the experimental procedures of this study.

2.2. Construction of the hepcidin expression adenovirus (ad-hepcidin)

Hepdicin protein encoding region (Genebank NM-053469) was cloned from the rat cDNA by polymerase chain reaction (PCR), and the recombined adenovirus named 'ad-

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☆☆ Conflict of interest: The authors declare that they have no conflict of interest.

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hepcidin' was constructed as Du et al. [16]. The virus with the titer of 3×10^9 optical particle units/μl were obtained for the infection of J774 cells. GFP-expression adenoviruses (ad-blank) were used as negative control.

2.3. Construction of the siRNA against hepcidin (retro-hepcidin)

pSUPER.retro.puro vector (OligoEngine, USA) was used for expression of siRNA targeting on hepcidin. The target sites for siRNA were designed by using an OligoEngine tool. The selected sequences were submitted to a BLAST search to avoid targeting the other nonspecific genome. 19-Nucleotide sequences (GTCTCTGTTGCA-TAACATA) corresponding to nucleotides 148–166 and 190–208 for hepcidin were selected to generate the pSUPER-hepcidin plasmid (vector). A control vector (pSUPER-Control) was constructed using a 19-nucleotide sequence (GCGCGCTTTGTAGGATTCG) with no significant homology to any mammalian gene sequence and therefore serves as a nonsilencing control (OligoEngine, USA). The nucleotide sequences were inserted into the pSUPER.retro vector after digestion with *Bgl*II and *Hin*dIII and transformed into One Shot TOP10 Chemically Competent E. Coli (Invitrogen, USA). Several clones were obtained and amplified, and selected positive clones were further verified by DNA sequencing. Retroviruses expressing hepcidin were produced by transfecting pSUPER-hepcidin plasmid into Phoenix Ampho packaging cell line according to the manufacturer's instructions. The absorbance of virus solution was read repeatedly at 260-nm wavelength, and the titration of virus was calculated by a formula: absorbance (260 nm)×multiple of dilution×1.1×10^{12}/ml.

2.4. Real-time PCR

Total RNA was isolated from J774 cells using TRIzol Reagent and then reverse transcribed into cDNA by using a Superscript II reverse transcriptase (Invitrogen, USA). Real-time PCR was carried out using iQ SYBR Green Supermix Kit and Bio-Rad iCycle system (Bio-Rad, USA), which used specific pairs of primers for hepcidin (forward primer, 5′-gaaggcaagatggcactaagca-3′; reverse primer, 5′-tctcgtctgttgccggagatag-3′) and β-actin (forward primer, 5′-gaaatcgtgcgtgacattaaagag-3′; reverse primer, 5′-gcggcagtggccatctc-3′). The hepcidin mRNA level of each sample was normalized to that of the β-actin mRNA. Relative mRNA level was presented as 2[control Ct(β-actin-hepcidin)−expt. Ct(β-actin-hepcidin)]. The specific band of hepcidin was confirmed by DNA gel electrophoresis [16].

2.5. Western blot analysis

The J774 cells received different treatments and were washed with ice-cold PBS, homogenized with lysis buffer and then sonicated using Soniprep 150 (MSE Scientific Instruments, London, UK). Protein content was determined using the Bradford assay kit (Bio-Rad). Aliquots of the total cell extract containing about 20 μg of protein were loaded on a single track of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and subsequently transferred to a pure nitrocellulose membrane (Bio-Rad). Molecular weight standards were run in parallel. The blots were blocked and then incubated with primary antibodies: mouse anti-rat TfR1 monoclonal antibody (1:1000, BD Transduction Laboratories), rabbit anti-rat DMT1+IRE, DMT1-IRE polyclonal antibodies and rabbit anti-mouse Fpn1 polyclonal antibody (1:5000, Alpha Diagnostic International Company). After three washes, the blots were incubated with goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody (1:5000, Li-Cor) for 1 h at room temperature. The intensity of the specific bands was detected and analyzed by Odyssey infrared imagine system (Li-Cor). To ensure even loading of the samples, the same membrane was probed with rabbit anti-rat β-actin polyclonal antibody at a 1:2000 dilution.

2.6. Measurement of Transferrin-Bound Iron (Tf-Fe) and Non-Transferrin-Bound Iron (NTBI) uptake

The cells with a concentration of 5×10^5 cells/ml were incubated with 0, 10, 30, 60 or 90 multiplicities of infection (MOI) of ad-hepcidin or 0 or 700 nM of hepcidin (Peptides International) at 37°C for 0, 0.5, 1, 3, 6, 9, 12, 18, 24, 36 or 48 h in DMEM medium containing 5% FBS for 16 h. The cells were then incubated with 2 μM of transferrin-bound iron (^{55}Fe-Tf) in 0.5 ml serum-free DMEM medium at 37°C for 30 min with gentle shaking. The ^{55}Fe-Tf solution was prepared, and the uptake of Tf-Fe by the cells was determined as previously described [17].

The radiolabeled ^{55}Fe (NTBI) solution was prepared as previously described [17]. The J774 cells with a concentration of 5×10^5 cells/ml were incubated with 0, 10, 30, 60 or 90 MOI of ad-hepcidin or 0 or 700 nM of hepcidin at 37°C for 0, 0.5, 1, 3, 6, 9, 12, 18, 24, 36 or 48 h in DMEM medium containing 5% FBS for 16 h. After that, the cells were incubated with 1.8 μM $^{55}FeCl_3$ in 0.5 ml serum-free DMEM medium at 37°C for 30 min with gentle shaking. The measurement of NTBI was then conducted [17].

2.7. Iron release assay

Fe release was measured as previously described [18]. Briefly, the J774 cells were incubated with 0 or 10 μM of ^{55}Fe solution at 37°C for 60 min and then with 0, 10, 30, 60 or 90 MOI of ad-hepcidin or 0 or 700 nM of hepcidin at 37°C for 0, 0.5, 1, 3, 6, 9, 12, 18, 24, 36 or 48 h. After that, the medium was collected and the cells were lysed. The radioactivity of both fractions was assayed by a scintillation counter (Perkin-Elmer) as cpm. The percentage of ^{55}Fe (II) release was calculated with to the following equation: ^{55}Fe (II) release (%)=[(cpm in medium)/(cpm in medium+cpm in cells)]×100%. Each treatment group was normalized with the individual control group.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA) for hepcidin and Protein Kinase A (PKA)

The expression of hepcidin and PKA in J774 cells and the amount of hepcidin released from astrocytes were assayed with ELISA kits (R&D Systems, MN, USA). Briefly, cells that were at a concentration of 1×10^6 cells/ml after the different treatments were collected and lysed by repeating freeze–thaw cycles for three times in 100 μl PBS and centrifugation at 2500 rpm for 20 min to determine hepcidin expression in the cells. The supernatants (10 μl) were used to determine the hepcidin and PKA levels according to the manufacturer's protocol. For measurement of hepcidin release, hepcidin level in the culture medium was determined. Briefly, the medium was collected and centrifuged for 20 min at a speed of 3000 rpm to remove the pellet. A volume of 10 μl of supernatant was sampled for measuring hepcidin according to the manufacturer's protocol.

2.9. Cyclic adenosine monophosphate (cAMP) assay

cAMP formation was assayed as described previously [19]. In brief, [2-3H]-adenine-labeled cells were washed once with 1 ml of 20 mM Hepes-buffered DMEM containing 1 mM isobutyl methylxanthine and then incubated with 1 ml assay medium at 37°C for 30 min in the presence of various concentrations of hepcidin with or without forskolin. Intracellular [3H]cAMP was obtained by sequential chromatography, and the ratio of [3H]cAMP to total [3H]ATP and [3H]ADP pools was calculated.

2.10. Statistical analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0) (SPSS, Inc., Chicago, IL, USA). Data were presented as mean±S.E.M. The difference between or among the means was determined by Kruskal–Wallis test followed by Mann–Whitney test for multiple comparisons or one-way or two-way analysis of

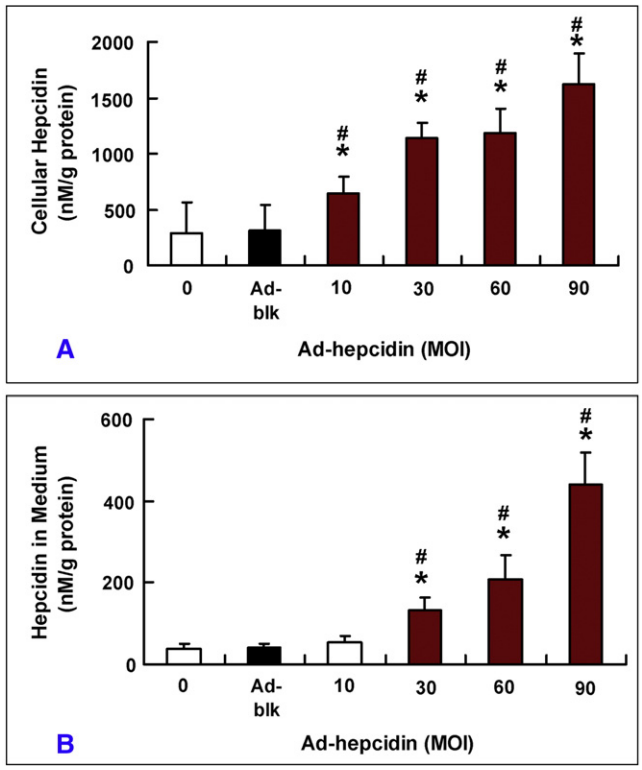

Fig. 1. Ad-hepcidin significantly increased expression and release of hepcidin peptide in J774 cells. The cells were infected with 0, 10, 30, 60 or 90 MOI of ad-hepcidin and ad-blank (Ad-b, 30 MOI) for 16 h, and the expression and release of hepcidin peptide were then determined by a ELISA. (A) Intracellular hepcidin peptide and (B) hepcidin peptide in the medium. Data are mean±S.E.M. (n=6). *P<.05; **P<.01 vs. the control.

variance in appropriate experiments followed by Newman–Keuls post hoc test. A probability value of $P<.05$ was taken to be statistically significant.

3. Results

3.1. Ad-hepcidin increases hepcidin expression and release in J774 macrophages

Infection of cells with ad-hepcidin induced a significant increase in cellular hepcidin protein as well as the content of hepcidin in the cultured medium both in a dose-dependent manner (Fig. 1). The findings indicated that infection with ad-hepcidin could increase not only expression but also release of hepcidin from the cells into the medium.

3.2. Ad-hepcidin inhibited Tf-Fe and NTBI uptake, iron release, and expression of the cellular iron uptake and release proteins in J774 cells

Treatment with ad-hepcidin induced a remarkable decrease in Tf-Fe uptake at the doses of 10, 30, 60 and 90 MOI (Fig. 2A); NTBI at 30, 60 and 90 MOI (Fig. 2B); and iron release at 60 and 90 MOI (Fig. 2C) in a dose-dependent manner. In addition, infection with ad-hepcidin resulted in a significant decrease in TfR1 expression at the doses of 10, 30, 60 and 90 MOI (Fig. 2D and E); DMT1+IRE at 30, 60 and 90 MOI (Fig. 2F and G); DMT1-IRE at 10, 30, 60 and 90 MOI (Fig. 2H and I); and Fpn1 at 60 and 90 MOI (Fig. 2J and K). The effect of ad-hepcidin on Tf-Fe uptake closely matches the effect on TfR1 expression in terms of pattern and degree. Also, the effects on NTBI and iron release were

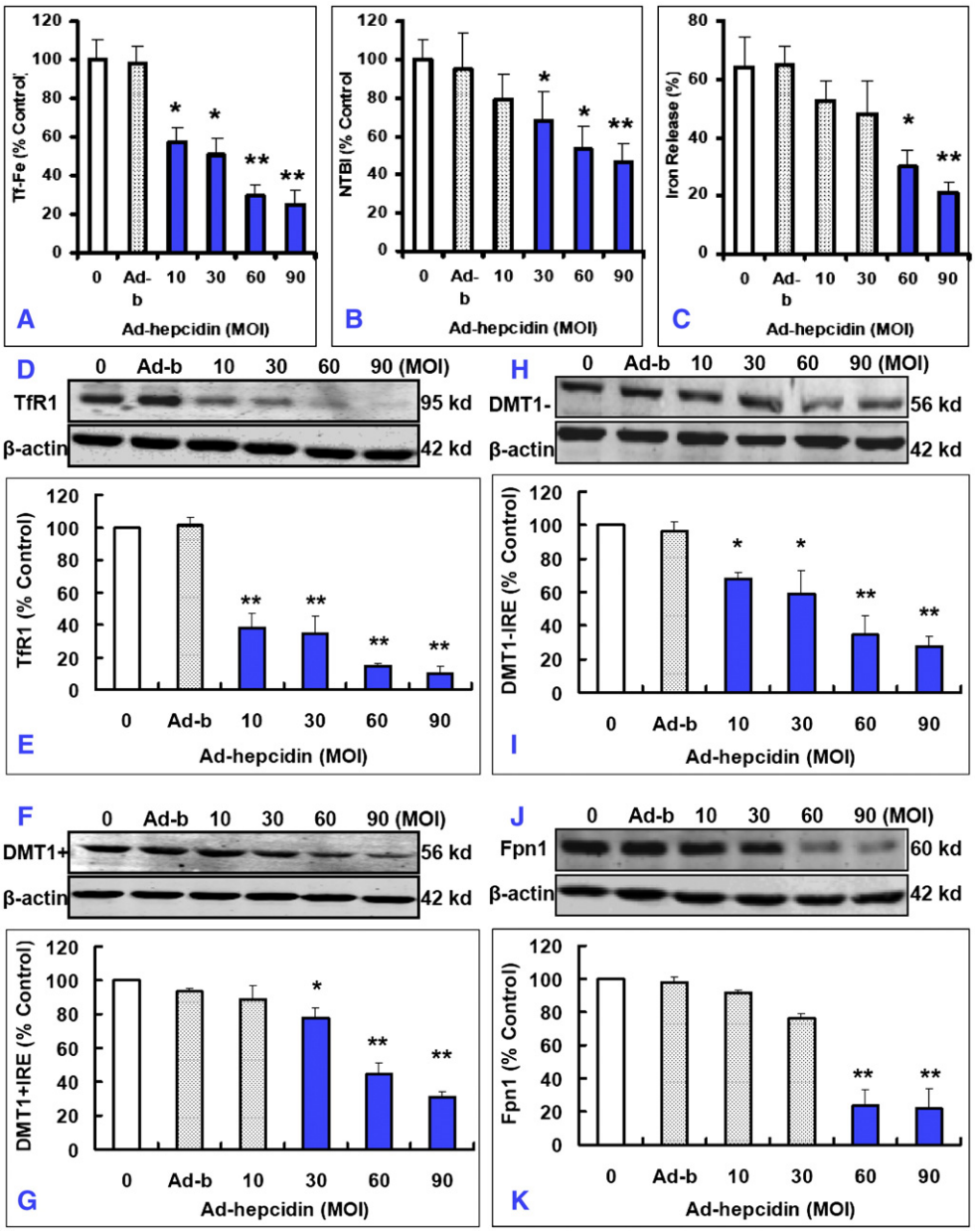

Fig. 2. Ad-hepcidin significantly inhibited iron uptake and release, and expression of iron transport proteins in J774 cells. (A) Tf-Fe uptake and (B) NTBI uptake: the cells were pretreated with 0, 10, 30, 60 or 90 MOI of ad-hepcidin and ad-blank (ad-b, 30 MOI) for 16 h and then incubated with or without ^{55}Fe-Tf (10 μg/ml) or ^{55}Fe(II) (1 μM) at 37°C for 30 min. (C) Iron release. The cells were incubated with ^{55}Fe(II) and then incubated with 0, 10, 30, 60 or 90 MOI of ad-hepcidin or ad-blank (ad-b, 30 MOI) for 16 h. The iron uptake (A and B) and release (C) were then measured using a radioisotope method. (D–K) (D and E: TfR1; F and G: DMT+IRE; H and I: DMT1-IRE; and J and K: Fpn1) The cells were treated with 0, 10, 30, 60 or 90 MOI of ad-hepcidin or ad-blank (ad-b, 30 MOI) for 16 h, and the expression of TfR1, DMT1+IRE, DMT1-IRE and Fpn1 was then determined by Western blot analysis. Data are mean±S.E.M. ($n=6$). *$P<.05$; **$P<.01$ vs. the control.

very similar to those on DMT1 and Fpn1 expression, respectively. These findings implied that the significant inhibitory role of ad-hepcidin on iron uptake and release is likely to be mediated by its inhibitory effect on the expression of these major proteins involved in iron uptake and release.

3.3. Hepcidin peptide inhibited Tf-Fe and NTBI uptake and iron release, and expression of the cellular iron uptake and release proteins in J774 cells

To confirm that the effects of ad-hepcidin on iron uptake and release are due to its ability to up-regulate expression of hepcidin peptide, we next investigated the effects of synthetic human hepcidin peptide on Tf-Fe and NTBI uptake and iron release, and also the expression of TfR1, DMT1+IRE, DMT1-IRE and Fpn1 in J774 cells. Hepcidin peptide at 700 nM induced a significant decrease in Tf-Fe (Fig. 3A), NTBI uptake (Fig. 3B) and iron release (Fig. 3C) as well as in the expression of TfR1 (Fig. 3D and E), DMT1+IRE (Fig. 3F and G), DMT1-IRE (Fig. 3H and I) and Fpn1 (Fig. 3J and K). These findings were completely consistent with those obtained from the ad-hepcidin experiments and provided direct evidence that ad-hepcidin plays its role by increasing expression of hepcidin peptide. Also, hepcidin peptide inhibits both Tf-Fe uptake (Fig. 3A) and TfR1 expression (Fig. 3D and E) in a time-dependent way within the 48-h observation period. However, the time-dependent inhibition of hepcidin on NTBI (Fig. 3B) and iron release (Fig. 3C) was found only at the earlier stage (0–6 or 9 h). A very similar effect of hepcidin peptide on the expression of DMT1 (Fig. 3F, G, H and I) and Fpn1 (Fig. 3J and K) was also observed. Highly significant correlations were found between TfR1 expression and Tf-Fe uptake (Fig. 3L), DMT1+IRE or DMT1-IRE expression and NTBI uptake (Fig. 3M and N) in J774 cells treated with hepcidin peptide.

3.4. The reduced hepcidin induced by retro-hepcidin leads to a significant increase in expression of iron uptake and release proteins in J774 cells

We also investigated the effects of retro-hepcidin on the expression of both iron uptake and release proteins in J774 cells by preincubating cells with retro-hepcidin (10 MOI) in DMEM for 48 h

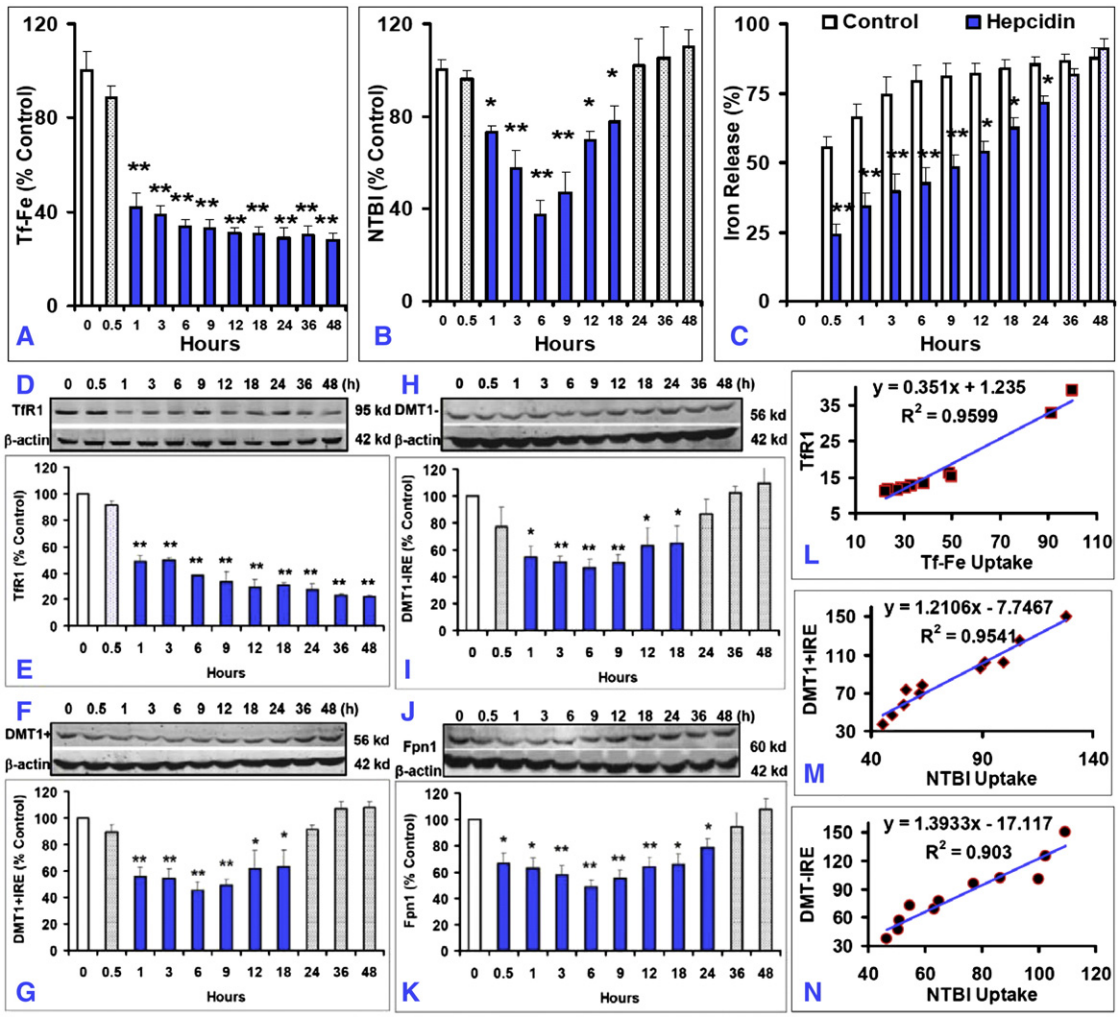

Fig. 3. Hepcidin peptide significantly inhibited iron uptake and release, and expression of iron transport proteins in J774 cells. (A) Tf-Fe uptake and (B) NTBI uptake: the cells were pretreated with 0 or 700 nM of hepcidin peptide for 0, 0.5, 1, 3, 6, 9, 12, 18, 24, 36 or 48 h and then incubated with ^{55}Fe-Tf (0 or10 μg/ml) or ^{55}Fe(II) (0 or 1 μM) at 37°C for 30 min. (C) Iron release: the cells were incubated with ^{55}Fe(II) (1 μM) for 30 min and then incubated with 0 or 700 nM of hepcidin peptide for 0, 0.5, 1, 3, 6, 9, 12, 18, 24, 36 or 48 h. The iron uptake (A and B) and release (C) were then measured using a radioisotope method. (D–K) (D and E: TfR1; F and G: DMT+IRE; H and I: DMT1-IRE; and J and K: Fpn1) The cells were treated with 0 or 700 nM of hepcidin peptide for 0, 0.5, 1, 3, 6, 9, 12, 18, 24, 36 or 48 h, and expression of the iron transport proteins was then determined. Data are mean±S.E.M. (n=8). *P<.05; **P<.01 vs. the control. (L–N) There are highly significant correlations between TfR1 expression and Tf-Fe uptake (L), DMT1+IRE (M) or DMT1-IRE (N) expression and NTBI uptake in J774 cells.

before the real-time PCR and Western blot analysis were conducted. The findings of the real-time PCR demonstrated that the incubation of neurons with retro-hepcidin induced a significant decrease in the expression of hepcidin (unpublished data). Western blot analysis showed that treatment with retro-hepcidin resulted in a significant increase in the expression of TfR1 (Fig. 3A and B), DMT1+IRE (Fig. 3C and D), DMT1-IRE (Fig. 3E and F) and Fpn1 (Fig. 3G and H).

3.5. The direct inhibitory effect of ad-hepcidin on the expression of cellular iron uptake proteins in J774 cells

The cells were pretreated with 2 mM of deferoxamine mesylate (DFO, Fe^{3+} chelator) and 0.5 mM of bathophenanthroline disulfonic acid (BP, Fe^{2+} chelator) in serum-free RPMI medium at 37°C for 16 h [20–23]. After washing, the cells were treated with 30 MOI of ad-hepcidin for 16 h, and expression of iron transport proteins was measured using Western blot analysis. The purpose of the pretreatment of the cells with Fe^{2+} and Fe^{3+} chelators was to deplete the cellular iron in order to exclude the indirect (cellular iron-mediated) inhibitory effect of hepcidin on the expression of these proteins (Fig. 4).

Treatment with DFO and BP induced a significant increase in the expression of iron uptake proteins. The levels of TfR1 (Fig. 5A and B), DMT1+IRE (Fig. 5C and D) and DMT1-IRE (Fig. 5E and F) in J774 cells treated with DFO and BP were significantly higher than those in the control cells. The posttreatment of the iron-depleted J774 cells with ad-hepcidin induced a significant decrease in the expression of these proteins. The contents of TfR1 (Fig. 5A and B), DMT1+IRE (Fig. 5C and D) and DMT1-IRE (Fig. 5E and F) in the iron-depleted J774 cells treated with ad-hepcidin were significantly lower than those in the iron-depleted cells without ad-hepcidin treatment. It appeared that the decreased expression of iron uptake proteins in the iron-depleted cells treated with ad-hepcidin should not be associated with the changes in cellular iron. In addition, treatment with DFO and BP induced a significant decrease in Fpn1 expression. The levels of Fpn1 in the iron-depleted cells were significantly lower than those in the control cells (Fig. 5G and H). The posttreatment of the iron-depleted cells with ad-hepcidin induced a further decrease in Fpn1 expression (Fig. 5G and H).

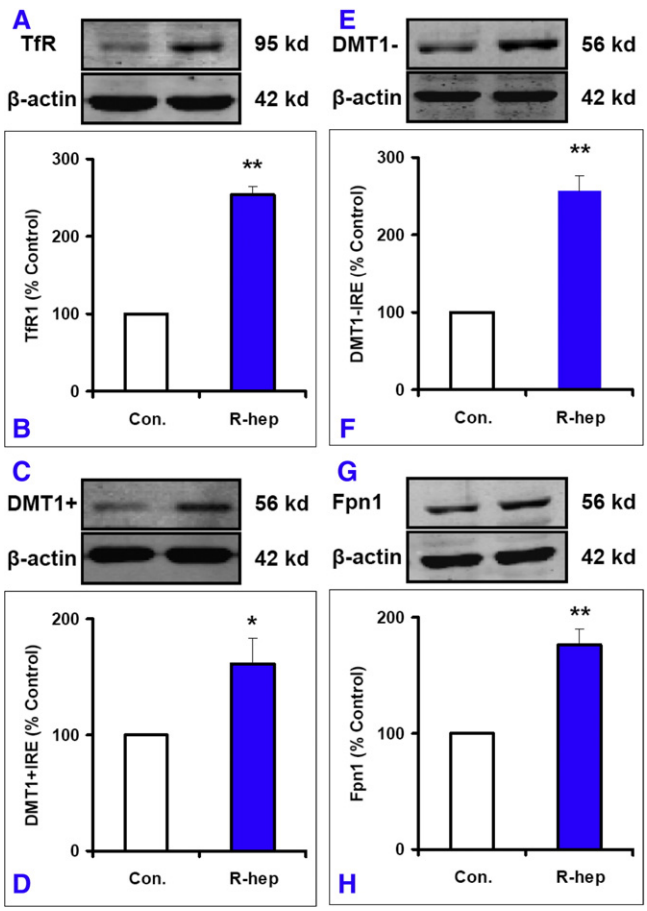

Fig. 4. Retro-hepcidin (siRNA against hepcidin) significantly increased expression of iron transport proteins in J774 cells. The cells were incubated with 0 or 10 MOI of retro-hepcidin (R-hep) for 48 h, and Western blot analysis was then conducted. (A and B) TfR1, (C and D) DMT1+IRE, (E and F) DMT1-IRE and (G and H) Fpn1 in J774 cells. Data are mean±S.E.M. (n=5). *P<.05, **P<.01 vs. the control.

3.6. The direct inhibition of hepcidin on the expression of TfR1 is associated with the cAMP–PKA pathway in J774 cells

The cells were incubated with or without 10 μM of Rp-cAMP (a competitive antagonist of cAMP binding to PKA), 200 μM of 8-bromo-cAMP (an activator of cAMP-dependent PKA) or 5 μM of forskolin (an activator of cAMP-dependent PKA) for 10 min and then infected with 30 MOI of ad-hepcidin for 24 h. After the treatments, the cells were lysed for Western blot analysis. It was found that Rp-cAMP induced a significant decrease in TfR1 (Fig. 5I and J) and PKA (Fig. 5Q and R), while 8-bromo-cAMP and forskolin led to a significant increase in TfR1 (Fig. 5I and J) and PKA (Fig. 5Q and R). The similar effects of Rp-cAMP, 8-bromo-cAMP and forskolin on TfR1 and PKA implied that the direct inhibition of hepcidin on TfR1 expression in the cells is likely to be associated with PKA expression. A significant decrease induced by Rp-cAMP and a significant increase by 8-bromo-cAMP and forskolin were also found in Fpn1 (Fig. 5O and P). The similar effects on Fpn1 and PKA might also imply that effects of hepcidin on Fpn1 expression in J774 cells are partly mediated by PKA expression. In the case of the expression of DMT1+IRE and DMT1-IRE, however, 8-bromo-cAMP, forskolin and Rp-cAMP did not induce significant effects on these proteins (Fig. 5K, L, M and N).

We also measured the effects of hepcidin on cAMP synthesis in J774 cells. The [2-3H]-adenine labeled J774 cells were incubated with 0, 700 or 1500 nM of hepcidin for 30 min or pretreated with 700 nM of hepcidin and then exposure to forskolin (0 and 50 μM) for 30 min at 37°C. The hepcidin (700 nM) significantly decreased basal cAMP level in J774 cells compared to the control where increasing the hepcidin concentration to 1500 nM did not show any further significant suppression of cAMP synthesis (Fig. 5S). The cAMP was significantly increased in forskolin-stimulated cells and measured about 2.5-folds compared to the control level. Pretreatment of cells with hepcidin before stimulation with 50 μM of forskolin significantly decreased the forskolin-stimulated cAMP level to where it becomes comparable to the basal level (Fig. 5T). These findings imply that the direct inhibition of hepcidin on the expression of TfR1 is associated with the cAMP–PKA pathway in J774 cells.

4. Discussion

On the cellular iron release protein Fpn1, hepcidin has a direct effect by directly binding with Fpn1 on the surface of macrophages and some other cells. However, little is known about the effects of hepcidin on cellular iron uptake proteins. The results on the expression of TfR1 in macrophages which received inflammatory stimuli are controversial. Inflammation generates various changes in body iron homeostasis, including iron sequestration in the reticulo-endothelial system [24–26]. Hepcidin could be markedly induced during inflammation. It has therefore been suggested that increased iron sequestration in the reticuloendothelial system or macrophages

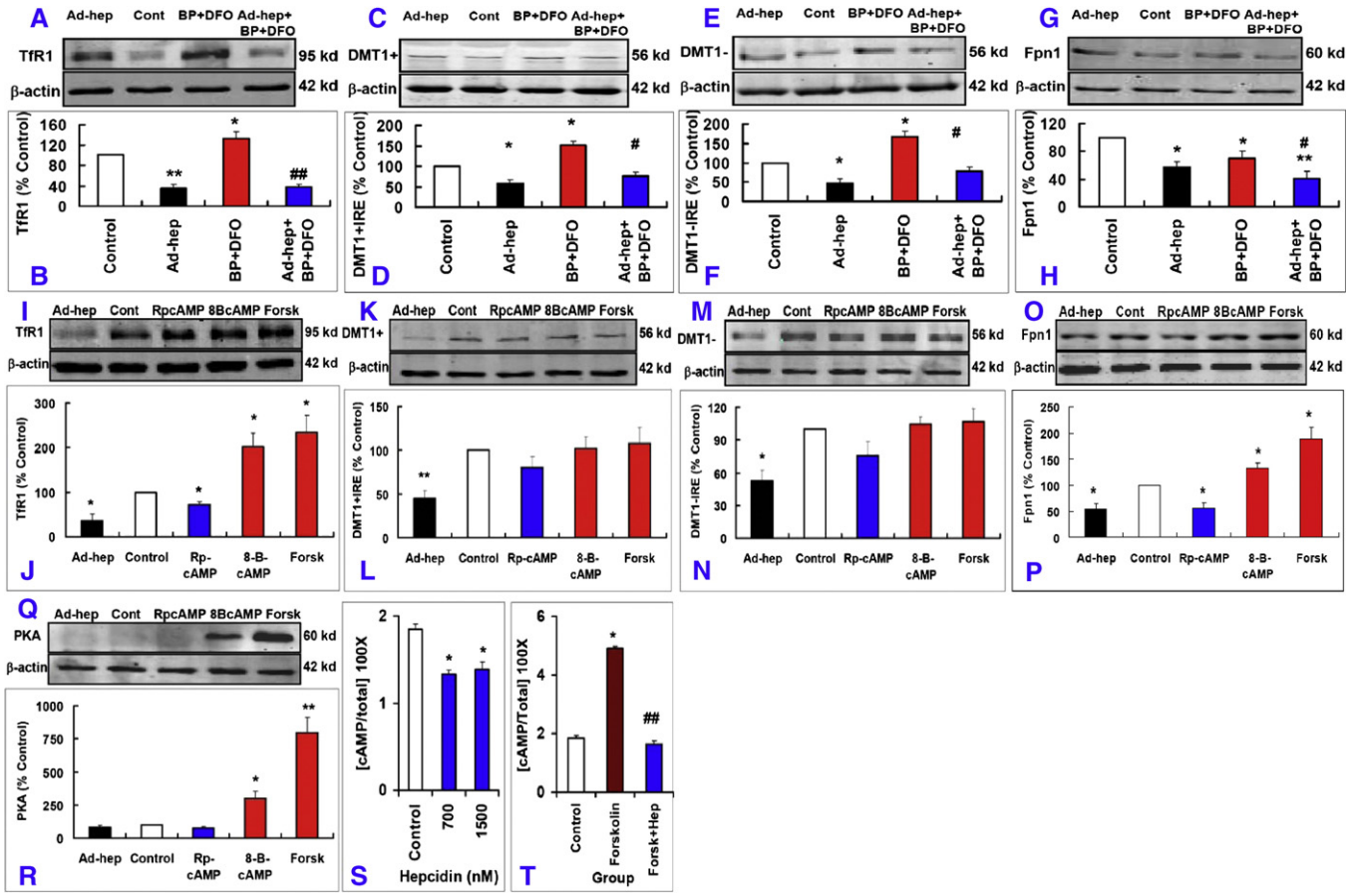

Fig. 5. (A–H) Ad-hepcidin significantly inhibited expression of cellular iron uptake proteins in the iron-depleted J774 cells (a direct inhibitory effect). (I–R) The effect of the activator or inhibitor of cAMP-dependent PKA on expression of iron transporter proteins and PKA in J774 cells. (S and T) Hepcidin peptide significantly reduced basal cAMP level in J774 cells. (A–H) Cellular iron was depleted by pretreatment with 2 mM of DFO (Fe3+ chelator) and 0.5 mM of BP (Fe^{2+} chelator) at 37°C for 16 h. After washing, the cells were treated with 0 or 30 MOI of ad-hepcidin for 16 h. (A and B) TfR1, (C and D) DMT1+IRE, (E and F) DMT1-IRE and (G and H) Fpn1. Data were mean±S.E.M. (n=5). *P<.05; **P<.01 vs. the control. $^{\#}P$<.05; $^{\#\#}P$<.01 vs. DFO+BP. (I–R) The cells were incubated with or without 10 μM of Rp-cAMP, 200 μM of 8-bromo-cAMP or 5 μM of forskolin for 10 min and then with 0 or 30 MOI of ad-hepcidin for 16 h. The cells were then lysed for Western blot analysis. (I and J) TfR1, (K and L) DMT1+IRE, (M and N) DMT1-IRE, (O and P) Fpn1, (Q and R) PKA. Data are mean±S.E.M. (n=5) *P<.05; **P<.01 vs. the control. (S and T) The [2-3H]-adenine-labeled J774 cells were incubated with 0, 700 or 1500 nM of hepcidin for 30 min (S) or pretreated with 700 nM of hepcidin and then exposure to 0 and 50 μM of forskolin (T) for 30 min at 37°C. The cAMP formation was then assayed. Data are mean±S.E.M. (n=4) *P<.05; **P<.01 vs. the control. $^{\#\#}P$<.01 vs. forskolin.

induced by inflammation is caused by hepcidin-mediated down-regulation of Fpn1 as well as up-regulation of TfR1 [26].

However, a number of studies of murine and human reticuloendothelial cells [25–32] have shown that TfR1 expression is posttranscriptionally down-modulated by exposure to inflammatory stimuli. Although they did not investigate the effects of inflammation on hepcidin, it is highly likely that the "down-regulation" of TfR1 expression found in these studies is at least partly due to the increased hepcidin induced by inflammatory stimuli. This possibility is strongly supported by our findings. We demonstrated that hepcidin peptide inhibited TfR1 expression and Tf-Fe uptake both in a time-dependent way. In addition, we demonstrated that another iron uptake protein, DMT1, was down-regulated and that NTBI uptake was significantly inhibited in the hepcidin peptide-treated cells. Our results suggested that increased iron sequestration in macrophages induced by inflammation is only due to the hepcidin-mediated down-regulation of Fpn1 and not associated with the effect of hepcidin on TfR1.

The data presented in this study provided the direct evidence for the first time that both ad-hepcidin and hepcidin peptide could significantly inhibit cellular iron uptake proteins TfR1 and DMT1 (−IRE and +IRE) expression as well as Tf-Fe and NTBI uptake. Correlation analysis of the relationship between the expression of proteins and iron transport demonstrated that there are highly significant correlations between TfR1 and Tf-Fe uptake, DMT1+IRE and NTBI uptake, and DMT1-IRE and NTBI uptake. In addition, it was found that retro-hepcidin induced a significant increase in TfR1 and DMT1 (DMT1+IRE and DMT1-IRE) expression as well as Tf-Fe and NTBI uptake (data not shown). These demonstrated that the reduced uptake of Tf-Fe and NTBI induced by hepcidin resulted from the down-regulation of TfR1 and DMT1 expression.

It is unknown whether the inhibition on the expression of iron uptake proteins is the direct effect of hepcidin in macrophages. The hepcidin-mediated down-regulation of Fpn1 would lead to an increase in the accumulation of cellular iron [26] and then inhibit the expression of iron uptake proteins including TfR1 by the IRE/IRP mechanisms [33]. Therefore, the reduced expression could be only due to the indirect (cellular iron-mediated) inhibitory effect of hepcidin. However, in the present study, we found that ad-hepcidin is still able to induce a significant decrease in the expression of TfR1, DMT1-IRE and DMT1+IRE in the iron-depleted J774 cells. In iron-depleted cells, hepcidin can induce a down-regulation of Fpn1 but will not be able to lead to a secondary increase in cellular iron. Therefore, this finding might suggest the existence of the direct inhibitory effect of hepcidin on the expression of cellular iron uptake proteins in J774 cells.

The possible existence of the direct inhibitory effect led us to speculate that there might be a novel unidentified receptor for hepcidin in the cells. In general, the receptors for the peptide hormones are located on the surface of target cell membrane, and the response of the target cells to peptide hormone is mediated by the second messengers including cAMP, Ca2+, tyrosine kinase and others [34]. In the present study, we found that Rp-cAMP, a competitive antagonist of cAMP binding to PKA, induced a decrease in PKA as well as TfR1, while 8-bromo-cAMP and forskolin, two activators of cAMP-dependent PKA, led to a significant increase in PKA and TfR1 in the cells. We also demonstrated that hepcidin significantly decreased and forskolin significantly increased cAMP levels in the cells. In addition, pretreatment with hepcidin could effectively prevent the effect of forskolin on cAMP levels in the cells. These findings imply that the direct inhibition of hepcidin on TfR1 expression might be mediated by the cAMP–PKA pathway in J774 cells. Hepcidin might be bound to the novel proposed receptor on the membrane and induce a decrease in cAMP and then PKA, leading to a significant decrease in transcription of TfR1.

In summary, we demonstrated for the first time that hepcidin has a direct inhibitory effect on the expression of iron transport proteins TfR1, DMT1+IRE and DMT1-IRE and iron release protein Fpn1 and therefore significantly reduces the uptake of Tf-Fe and NTBI and iron release in J774 macrophages. We also showed that the direct inhibitory effect on TfR1 is associated with cellular cAMP and PKA, probably being mediated by a cAMP–PKA pathway in J774 macrophages.

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