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The iron regulatory hormone hepcidin reduces ferroportin 1 content and iron release in H9C2 cardiomyocytes

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Abstract

Iron plays a key pathophysiological role in a number of cardiac diseases. Studies on the mechanisms of heart iron homeostasis are therefore crucial for understanding the causes of excessive heart iron. In addition to iron uptake, cellular iron balance in the heart also depends on iron export. We provided evidence for the existence of iron exporter ferroportin 1 (Fpn1) in the heart in a recent study. The presence of hepcidin, a recently discovered iron regulatory hormone, was also confirmed in the heart recently. Based on these findings and the inhibiting role of hepcidin on Fpn1 in other tissues, we speculated that hepcidin might be able to bind with, internalize and degrade Fpn1 and then decrease iron export in heart cells, leading to an abnormal increase in heart iron and iron mediated cell injury. We therefore investigated the effects of hepcidin on the contents of Fpn1 and iron release in H9C2 cardiomyocyte cell line. We demonstrated that hepcidin has the ability to reduce Fpn1 content as well as iron release in this cell. The similar regulation patterns of hepcidin on the Fpn1 and iron release suggested that the decreased iron release resulted from the decreased content of Fpn1 induced by hepcidin. We also found that hepcidin has no significant effects on ceruloplasmin (CP) and hephaestin (Heph) — two proteins required for iron release from mammalian cells. The data imply that Fpn1, rather than Heph and CP, is the limited factor in the regulation of iron release from heart cells under physiological conditions.

Keywords: Hepcidin; H9C2 cardiomyocytes; Ferroportin 1; Hephaestin; Iron release; Heart iron

1. Introduction

Iron is an essential trace element for a variety of metabolic processes, but it may also cause potentially deleterious effects in humans [1]. The redox ability of iron can lead to the production of oxygen free radicals, which can damage various cellular components. For this reason, the iron levels in tissues must be tightly regulated. The increased iron and iron-mediated injury have been proposed to play an important role in the development of a number of heart disorders, including heart ischemia-reperfusion injury, iron-overload cardiomyopathy, acute myocardial infarction and coronary heart diseases [2–8]. At present, it is unknown how iron increases to a pathological level in the heart under abnormal circumstances. Study on how myocardial iron content is regulated is therefore crucial to understand this key issue.

In addition to iron uptake and storage, cellular iron balance in the heart also depends on the amount of iron

Abbreviations: CP, ceruloplasmin; Fpn1, ferroportin 1; Heph, hephaestin; IREG1, iron-regulated transporter 1; JH, juvenile haemochromatosis; MTP1, metal transport protein 1; NTBI, non-transferrrin-bound iron.

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export from the cells under physiological conditions. Recently, we investigated expression of iron exporters including ferroportin 1 (Fpn1), ceruloplasmin (CP) and hephaestin (Heph) and provided evidence for their existence in the heart [8]. We also demonstrated that iron has a significant effect on expression of Fpn1 and CP. The existence of these proteins and the effect of iron on their expression in the heart imply that they might play a role in heart iron homeostasis, which involves iron release from the heart cells. A recent study [1] also demonstrated that hepcidin, a recently discovered iron regulatory hormone [9,10], is expressed in the heart. They found that hepcidin is detectable in the heart although it is predominantly expressed in the liver. An important role of hepcidin in the development of cardiac diseases has been suggested [1].

Accumulated data have indicated that hepcidin is a principal regulator in the maintenance of systemic iron homeostasis [11,12]. Hepcidin controls plasma iron concentration and tissue distribution of iron by inhibiting intestinal iron absorption, iron recycling by macrophages and iron mobilization from hepatic stores [13]. It has also been confirmed that the inhibiting role of hepcidin is initiated by a direct binding of this peptide with Fpn1 on the surface of absorptive enterocytes, macrophages, hepatocytes and placental cells [14–16]. The complex of hepcidin and Fpn1 is then internalized and degraded, leading to a decreased export of cellular iron [17,18]. By this mechanism, hepcidin inhibits dietary iron absorption, the efflux of recycled iron from the reticuloendothelial macrophages and the release of iron from storage in hepatocytes. Based on the inhibiting role of hepcidin on Fpn1 in the above tissues, the existence of this peptide as well as Fpn1 in the heart, the potential connection between the mutations in the hepcidin and/or hemojuvelin genes and the severe heart failure in juvenile haemochromatosis [19], we speculated that hepcidin might be able to bind with, internalize and degrade Fpn1 and then decrease iron export in the heart cells. In this study, we therefore investigated the effects of hepcidin on the contents of Fpn1 as well as Heph and CP and iron release in H9C2 cell, a cardiomyocytes cell line. We demonstrated for the first time that hepcidin has the ability to reduce Fpn1 content as well as iron release in this cell line. The similar regulation patterns of hepcidin on the Fpn1 content and iron release suggested that the decreased iron release resulted from the decreased content of Fpn1 induced by hepcidin.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA. $^{55}FeCl_3$ obtained from Perkinelmer Company, Wellesley, MA, USA. Prestained protein marker was bought from Bio-Rad Laboratories, Hercules, CA, USA. The antibodies against Fpn1 and Heph were purchased from Alpha Diagnostic International Company, San Antonio, TX, USA, and the mouse antirat CP IgG1 and CD71 monoclonal antibodies from BD Transduction Laboratories, Franklin Lakes, NJ, USA. The human synthetic 25 amino acids hepcidin was obtained from Peptides International, Louisville, KY, USA. The concentrations of the hepcidin used were 70 and 700 nM according to the studies of Park et al. [20], Nemeth et al. [17] and Rivera et al. [21].

2.2. H9C2 cardiomyocyte culture

The H9C2 cardiomyocytes (a permanent cell line derived from the embryonic rat ventricle [22]) were obtained from the American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium (Glutamax; Life Technologies), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml of sodium penicillin G and 100 μg/ml of streptomycin sulfate. The medium was changed every 3 days. The subculture was prepared by removing the medium, and adding 1-3 ml of fresh 0.25% trypsin or 2mM EDTA solution (for protein extraction) for several minutes. The culture was allowed to stand at room temperature for 10 to 15 min. Fresh medium was added, aspirated and dispensed until the cells were detached. Then the cells were transferred to a 15 ml centrifuge tube containing 3–5 ml fresh medium and centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was discarded, and the pellet was triturated in 2 ml fresh medium. The cell number was determined by trypan blue exclusion under the microscope, and the required number of cells was placed into flasks (for maintenance) or in six-well plates and 35-mm culture dishes. All the apparatus and mediums used for cell culture were sterilized before use.

2.3. Western blot analysis

To determine the effect of hepcidin on Fpn1 and Heph expression or content in H9C2 cardiomyocytes, the cells were treated with hepcidin (0, 70 or 700 nm) for 0, 8, 16 or 24 h at 37°C. The cells receiving different treatments were homogenized in RIPA (radio immunoprecipitation assay) buffer and then sonicated using Soniprep 150 (MSE Scientific Instruments, London, UK) for 3×10 s [23]. After centrifugation at 10,000×*g* for 15 min at 4°C, the supernatant was collected. Protein content was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). A total of 30 μg of protein was diluted in 2× sample buffer (50 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS-polyacrylamide gel electrophoresis on 10% or 7.5% gel and subsequently transferred to a PVDF membrane (polyvinylidene difluoride; Bio-Rad). The membrane was then blocked with 5% blocking milk (Bio-Rad) in TBS containing 0.1% Tween 20 for 2 h at room temperature. The membrane was rinsed in three changes of TBS-T, incubated once for 15 min and twice for 5 min in fresh washing buffer and incubated with primary

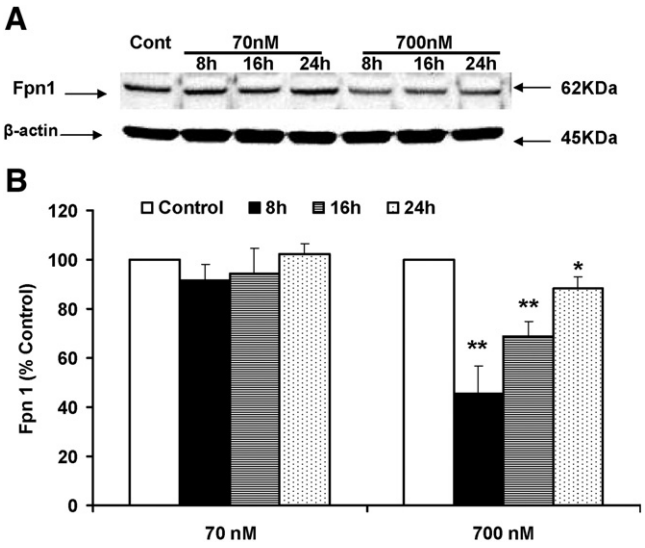

Fig. 1. Effects of hepcidin on the level of Fpn1 protein in H9C2 cardiomyocytes. The cells were treated with 0, 70 or 700 nM of hepcidin for 0 (the control), 8, 16 and 24 h, respectively. Western blot analysis was then conducted. (A) Representative Western blot of Fpn1 (~62 kDa). (B) The relative values of Fpn1 protein. Data were normalized by β-actin and expressed means±S.E.M. (% control, n=4). *P<.05; **P<.01 vs. the control.

antibody for 2 h at room temperature. The concentration of the rabbit antirat Fpn1 and Heph primary polyclonal antibody was 1:5000. After three washes, the membrane was incubated for 2 h in horseradish peroxidase-conjugated antirabbit and antimouse secondary antibody (1:5000, Amersham Biosciences) and developed using enhanced chemiluminescence (ECL Western blotting analysis system kit, Amersham Biosciences). The blot was detected using a Lumi-imager F1 workstation (Roche Molecular Biochemical). The intensity of the specific bands was determined by densitometry with the use of LumiAnalyst 3.1 software (Roche Molecular Biochemical). To ensure even loading of the samples, the same membrane was probed with mouse antirat β-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) at a 1:5000 dilution.

2.4. Iron release assay

The radio-labelled ^{55}Fe(II) solution was prepared and the Fe(II) (NTBI) uptake was measured as described previously [24,25]. Briefly, the ^{55}Fe(II) solution was prepared by mixing $^{55}FeCl_3$ and $^{56}FeSO_4$ in a molar ratio of 1:10, then adding 50-fold molar 2-mercaptoethanol and 0.27 M sucrose to give a final concentration of 62.5 μM. The incubation medium was the 0.27 M sucrose with 4 mM pipes, pH 6.5. After washing with phosphate-buffered saline (PBS) three times, the cells were incubated with ^{55}Fe(II) solution [2 (M) for 30 min at 37°C] and washed three times with PBS at 4°C. The cells were then incubated at 37°C for 0, 8, 16 or 24 h, with or without 700 nM of hepcidin. After that, the medium was collected and the cpm measured following centrifugation. The cells were detached by 500 μl lysis buffer. A 50-μl aliquot was subjected to the detection of the protein concentration. The cytosol was separated from the stromal fraction by centrifugation at 10,000×g for 20 min at 4°C. A 3-ml scintillation solution was added to both fractions to count the cpm. The sum of the radioactivity in the medium and in the cell (cytosol and stromal fractions) was named the total cellular radioactivity. The relative percentage of the total radioactivity in the medium and in the cell was calculated.

2.5. Statistical analysis

Statistical analyses were performed using SPSS software for Windows (version 10.0). Data were presented as mean±S.E.M. The difference between the means was determined by one-way analysis of variance followed by a Student-Newman-Keuls test for multiple comparisons. Differences with P<.05 were considered significant.

3. Results

3.1. Hepcidin decreases expression of Fpn1, but has no effect on Heph

To determine the effect of hepcidin on the synthesis of Fpn1 protein, the total protein in cell extracts derived from cultured H9C2 cells treated with hepcidin (0, 70 and 700 nM) for 0, 8, 16 or 24 h were analyzed using the Western blot. A single major band was observed with these antibodies with molecular weight (Mr) of ~62 kDa for Fpn1 or ~45 kDa for β-actin (Fig. 1A), which were in good agreement with the expected molecular weights based on

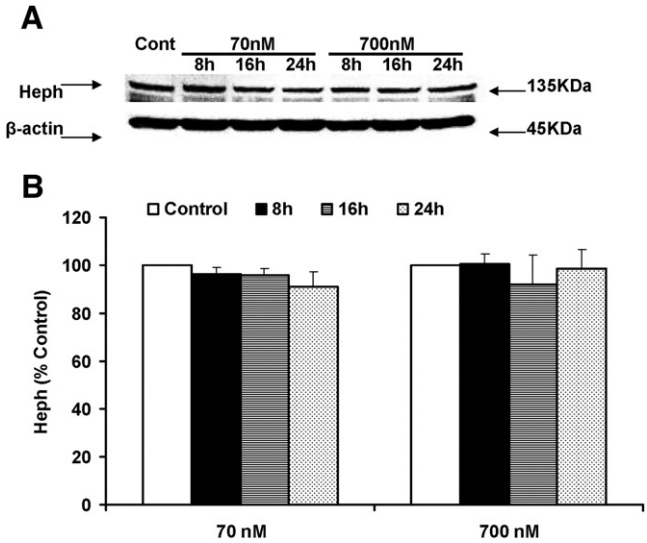

Fig. 2. Effects of hepcidin on the expression of Heph protein in H9C2 cardiomyocytes. The cells were treated with 0, 70 or 700 nM of hepcidin for 0 (the control), 8, 16 and 24 h, respectively. Western blot analysis was then conducted. (A) Representative Western blot of heph protein (~135 kDa). (B) The relative values of Heph protein. Data were normalized by β-actin and expressed means±S.E.M. (% control, n=4). No significant differences were found among the values.

published articles. As illustrated in Fig. 1B, 70 nM of hepcidin did not induce any significant effect on Fpn1, but 700 nM of hepcidin reduced significantly the expression or content of Fpn1. After incubation for 8 h, Fpn1 protein decreased markedly, with the level of Fpn1 dropping to as low as about 45% of the control. The level of Fpn1 protein then increased gradually with the incubation time, being about 68% and 88% of the control content after incubation for 16 and 24 h. The values in these two time points are still lower than the control level ($P<.05$).

Iron release from mammalian cells also requires Heph or CP. To investigate whether the expression of Heph or CP was also influenced by hepcidin, we used the western blot to measure the Heph and CP protein levels. We detected one expected band with a relative Mr of ~135 KDa for Heph (Fig. 2A) or ~150 KDa for CP (data not shown). Analysis of the band showed that the expression of Heph protein did not change significantly after treatment with 70 or 700 nM of hepcidin for 8, 16 or 24 h (Fig. 2B). Also, our data showed that hepcidin had no significant effect on the content of CP (data not shown). These findings might imply that Heph and CP are not the limited factors in the process of iron release from H9C2 cells under our experimental conditions.

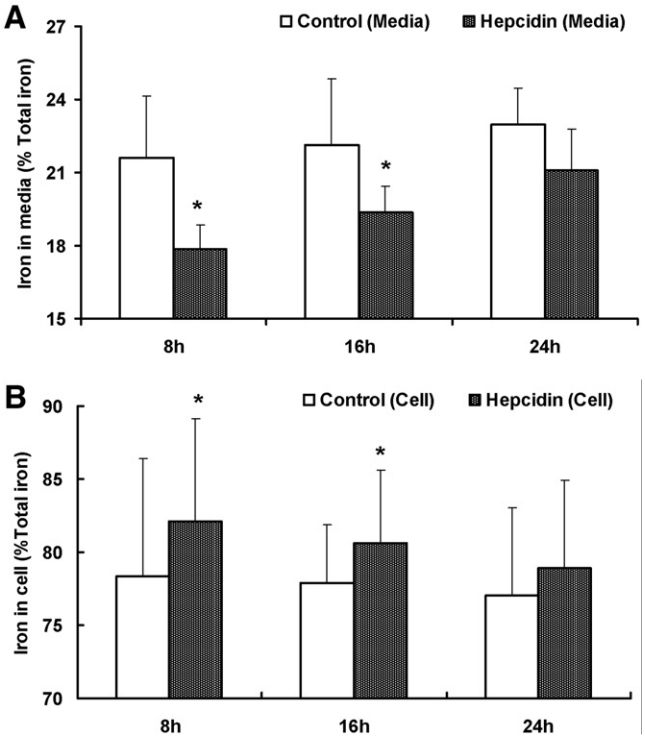

Fig. 3. Effects of hepcidin on iron release by H9C2 cardiomyocytes. The cells were preloaded with iron by incubation with 2 μM ^{55}Fe(II) in sucrose (pH 6.5) for 30 min at 37°C, then washed and incubated with or without 700 nM of hepcidin at 37°C for 0, 8, 16 or 24 h. Radiolabelled iron in the media and in the cell at different time points was respectively measured. Results were expressed as the percentage of total iron present in the system (radiolabelled iron in the media and cells). Data are means±S.E.M. (% total iron, n=6). *$P<.05$ vs. the control.

3.2. Hepcidin decreases iron release from H9C2 cardiomyocytes

We then examined whether the hepcidin-induced decrease in the content of Fpn1 would affect iron release from H9C2 cells. The cells were preloaded with 2 μM Fe(II) for 30 minutes and then washed three times with cold PBS solution, followed by incubation with or without 700 nM of hepcidin for 0, 8, 16 or 24 h. After the treatment, iron in the medium and the cells was respectively measured. It was found that exposure of iron-loaded H9C2 cells to hepcidin for 8 h induced a significant decrease in the radioactivity (iron release) in the medium (17.86% of the total iron, $P<.05$ vs. the control: 21.63% of total iron) (Fig. 3). Then, iron in the medium gradually increased with the incubation time. After incubation of the cells with 700 nm of hepcidin for 16 h, the radioactivity (iron release) in the medium was 19.34% of total iron, which is also significantly lower than that in the cells treated without hepcidin (22.13% of the total iron, $P<.05$). However, no significant differences in the radioactivity (iron release) in the medium were found between the experimental and control cells at the time point of 24 h. These were basically consistent with the changes in the expression or content of Fpn1 protein. The similarity of responses of Fpn1 and iron in the medium to hepcidin suggests that the decreased iron release from the cells results from the reduced Fpn1.

4. Discussion

To our knowledge, this is the first report about the effects of hepcidin on Fpn1 as well as iron release in cardiac cells. In this report, we demonstrated for the first time that hepcidin can significantly reduce Fpn1 expression or content as well as iron release in the cells. The response of Fpn1 content to the treatment with hepcidin was parallel to that of the amount of iron release from the cells. This suggested that the decreased iron release is a result of the decreased content of Fpn1 induced by hepcidin. Based on current knowledge on the mechanisms involved in the hepcidin-mediated inhibition on Fpn1 and iron release, it is possible that the inhibiting role of hepcidin on iron release in the cardiac cells might also be initiated by a direct binding of this peptide with Fpn1. After binding with hepcidin, Fpn1 is then internalized and degraded, leading to a decreased iron export from the cardiac cells as found in macrophages [17].

Fpn1 (or MTP1/REG1/Slc11a3) is a newly identified cellular iron exporter [14–16]. Fep1-mediated iron release from mammalian cells also requires ferroxidase Heph or CP [26,27]. It is widely believed that Heph or CP is necessary for ferrous iron (Fe^{2+}) to be oxidized to ferric iron (Fe^{3+}) after it crosses the cell membrane by a Fpn1-mediated process. The latter can then bind to the transport carrier transferrin. We therefore investigated whether hepcidin can also affect the expression of Heph or CP. Results showed that there is no

significant effect of hepcidin on Heph or CP expression. This finding, together with our recent study about the role of iron in the regulation of Fpn1, Heph and CP in the heart [8], implies that Fpn1, rather than Heph and CP, is the limited factor in the control of iron release from the heart cells.

In contrast to the hepcidin produced in the liver that plays a systemic effect, a recent study by Merle et al. [1] demonstrated that the hepcidin expressed in the heart is an intrinsic cardiac hormone and may have predominantly a local effect in the heart and that regulation of heart hepcidin by hypoxia is opposite to that in liver. They also showed that expression of hepcidin in the heart, like its expression in the liver, was significantly up-regulated under turpentine-induced inflammation [1]. The increased cardiac hepcidin in response to inflammation may be related to its antimicrobial function. The up-regulation of hepcidin can decrease iron level in local extra-cellular fluid and then restrict the availability of iron to invading microorganisms in the heart. However, based on the findings from the present study, the increased hepcidin in the heart will also be able to induce a decrease in Fpn1 expression or content as well as iron release from cardiac cells, then leading to an increase in intracellular iron concentration and iron-mediated free radical reaction. It might be partly associated with the development of heart cell injury in some cardiac diseases induced by microorganisms.

Our data also showed that the lowest content of Fpn1 was observed at 8 h of incubation of the cells with hepcidin. After this time point, the Fpn1 level progressively increased with the incubation time. Without doubt, the progressive increase in Fpn1 level at the late stage (8–24 h) is mainly due to the gradual decrease in hepcidin content in the media. However, it might also be associated with the gradually increased iron in the cells induced by the decreased iron release. It has been demonstrated that the mRNA transcript of Fpn1 contains an iron responsive element (IRE) in its 5′ untranslated region [14–16]. In an investigation on the functional role of genomic SLC40A1 elements (Fpn1 is a product of the SLC40A1 gene) in response to iron in human hepatoma (HepG2), intestinal carcinoma (Caco2) and lymphomonocytic (U937) cell lines, Lymboussaki et al. [28] demonstrated directly that the IRE in Fpn1 mRNA is functional and that it controls Fpn1 expression through the cytoplasmic IRP (iron regulatory protein). The findings in murine J774 macrophages and human alveolar macrophages also showed that there is a transcriptional mechanism that controls Fpn1 expression in these cells. These findings are in favor of the possibility that the gradually increased iron in the cells induced by the decreased iron release is partly responsible for the progressive increase of Fpn1 level at the late stage.

Although our study demonstrated that hepcidin can significantly reduce Fpn1 as well as iron release in cardiac cells, it is unknown how hepcidin regulates Fpn1 expression or content in cardiac cells. It also needs to be directly confirmed whether the decreased iron release is a result of the decreased Fpn1 induced by hepcidin as we suggested. In addition to the inhibiting role of hepcidin on iron exporter and iron release, it is completely unknown whether hepcidin plays a role to control iron uptake and expression of iron uptake proteins such as transferrin receptor and divalent metal transporter 1. The existence of these uptake proteins in the heart has been demonstrated [29]. The investigations of these questions will contribute to our knowledge about the physiological role of hepcidin in cardiac iron homeostasis and also provide insights into understanding how iron increases to a pathological level in the heart under abnormal circumstances.

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