Does Hepatic Hepcidin Play an Important Role in Exercise-Associated Anemia in Rats?

Yu-Qian Liu, Yan-Zhong Chang, Bin Zhao, Hai-Tao Wang, and Xiang-Lin Duan

Some athletes are diagnosed as suffering from sports anemia because of iron deficiency, but the regulatory mechanism remains poorly understood. It is reported that hepcidin may provide a way to illuminate the regulatory mechanism of exercise-associated anemia. Here the authors investigate the hepcidin-involved iron absorption in exercise-associated anemia. Twelve male Wistar rats (300 ± 10 g) were randomly divided into 2 groups, 6 in a control group (CG) and 6 in an exercise group (EG, 5 wk treadmill exercise of different intensities with progressive loading). Serum samples were analyzed for circulating levels of IL-6 by means of enzyme-linked immunosorbent assay (ELISA). The expression of hepatic hepcidin mRNA was examined by real-time polymerase chain reaction analysis. The protein levels of divalent metal transporter 1 (DMT1), ferroportin1 (FPN1), and heme-carrier protein 1 (HCP1) of duodenum epithelium were examined by Western blot. The results showed that the amount of iron and ferritin in serum were lower in EG than in CG (p < .05). The levels of IL-6 and white blood cells were greater in EG than in CG (p < .01). The expression of DMT1, HCP1, and FPN1 was significantly lower in EG than in CG (p < .01). The mRNA expressions of hepatic hepcidin and hemojuvelin in skeletal muscle were remarkably higher in EG than in CG. The data indicated that inflammation was induced by strenuous exercise, and as a result, the transcriptional level of the hepatic hepcidin gene was increased, which further inhibited the expression of iron-absorption proteins and led to exercise-associated anemia.

Keywords: iron absorption, inflammation, duodenum, skeletal muscle

Materials and Methods

Animals and Exercise Protocol

Male Wistar rats were used in all experiments. All animals were provided free access to food and distilled water. The Animal Care and Use Committee of Hebei Science and Technical Bureau in the People’s Republic
of China approved the experimental procedure. Twelve Wistar rats (300 ±10 g, purchased from Hebei Medical University, People’s Republic of China) were randomly assigned to two groups: a control group (CG) and an exercise group (EG, 5 weeks of treadmill running with different intensities and progressive load). The rats in CG remained sedentary and were placed on the treadmill without running. Exercise training consisted of running on a motorized treadmill for 5 weeks at 30 m/min (Harris, Mitchell, Sood, Webb, & Venema, 2008), 0% grade for 2 min (the first training), with the training time increased by 2 min each session for 6 days/week. The treadmill was always kept at 0% inclination. This speed–grade combination has been shown to yield slightly less than 80–85% of maximal oxygen consumption (VO₂max). The rats were trained once a day during the first 2 weeks and then twice a day for the next 3 weeks. The 5 weeks of training was less than the mean life span of human beings. The average lifespan of rats is 2–3 years, and of humans, ~70 years. Therefore, the relative training time is enough for experimental rats. Furthermore, decreased Hb showed that the exercise model was successful. Rats were housed in stainless steel cages at 22–24 °C, with relative humidity of 45–55%.

**Blood and Tissue Sampling**

The rats were fasted for 16 hr before blood sampling and were sacrificed 36 hr after the last exercise session. They were anesthetized with 0.4% pentobarbital sodium (1 ml/100 g body weight). Blood samples were collected in EDTA-K⁺-buffered tubes and taken immediately for determination of red blood cell (RBC) count, Hb concentration, and hematocrit (Hct) by an automatic blood analyzer (MEK-6318K, Japan). Blood samples were drawn into tubes with serum separator from the right auricle and then centrifuged within 2 hr for 15 min at 845 g at 4 °C (Liu, Duan, Chang, Wang, & Qian, 2006). Plasma was then aspirated, aliquotted, and frozen at −80 °C before it was assayed. The serum samples were analyzed for serum iron by using iron and iron-binding capacity reagent (Sigma). Plasma levels of IL-6 and serum ferritin were quantified by applying rat IL-6 kits (R&D Systems, Inc., Minneapolis, MN), rat serum ferritin kits (Immunology Consultants Laboratory, Inc., Newberg, OR, USA), and an enzyme-linked immunosorbent assay (ELISA) with the use of Gel-Pro Analyzer Analysis software (Media Cybernetics). To ensure even loading of the samples, the same membrane was probed with rabbit antirat β-actin polyclonal antibody (Sigma-Aldrich, St. Louis, MO) at 1:5,000 dilutions. Raw values obtained for DMT1, FPN1, and HCP1 were standardized to the β-actin endogenous control (Liu et al., 2006).

**Expression of DMT1, HCP1, and FPN1 in Rat Duodenum by Western Blot Analysis**

The duodenum was homogenized by supersonic in Tris-buffered salt (1 ml/100 mg). After the homogenate was centrifuged at 12,000 g for 30 min at 4 °C, the supernatant was collected. Protein concentration in supernatant was assayed by the Bradford method. The rabbit antirat DMT1 (non-IRE) antiserum, DMT1 (IRE) antiserum, FPN1 antiserum (ADI, San Antonio, TX), and HCP1 antiserum (Abcam) were 1:5,000. The immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences). The blots were detected by means of a chemiluminescence imaging system (Gene Snap Bio Imaging System). The intensity of the specific bands was determined by transmittance densitometry with the use of Gel-Pro Analyzer Analysis software (Media Cybernetics). To ensure even loading of the samples, the same membrane was probed with rabbit antirat β-actin polyclonal antibody (Sigma-Aldrich, St. Louis, MO) at 1:5,000 dilutions. Raw values obtained for DMT1, FPN1, and HCP1 were standardized to the β-actin endogenous control (Liu et al., 2006).

**Expression of Hepatic Hepcidin mRNA and HJV mRNA in Rat Gastrocnemius by Quantitative Real-Time Polymerase-Chain-Reaction Analysis**

Total RNA was isolated from liver and gastrocnemius using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was generated from 1 μg RNA using a reverse transcription kit (Promega) following the manufacturer’s instruction, and the reaction was performed at 70 °C for 10 min followed by 42 °C for 1 hr. Sequences of primers used to amplify rat mRNA by real-time polymerase chain reaction (PCR) were as shown in Table 1 (Christiansen et al., 2007, Zhang et al., 2007). PCR products were amplified (95 °C for 10 s fol-
followed by 40 cycles of 95 °C for 15 s and 60 °C for 31 s) by using a PCR mix (SYBRPremix Ex TaqTM Perfect Real Time, TaKaRa) and analyzed on a real-time PCR cycler, the ABI PRISM 7000 sequence-detection system (Applied Biosystems). For quantification, the fluorescence intensity was plotted against the PCR cycle number. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as the threshold cycle (CT). The CT value of each sample was compared with CT values of the standardization series, which consisted of cloned PCR fragments (Yamamoto, Otani, & Otsuki, 2006). The amounts of hepcidin and HJV transcripts were normalized to the amounts of β-actin transcripts in the same cDNA (expressed as fold change per β-actin). There were six samples in each group, and each sample was analyzed in duplicate. The real-time PCR experiments were performed three times, and all data were combined for analysis.

### Statistics

To avoid bias, the identities of experimental groups were assigned as numbers and not revealed to the laboratory experimenters. The results are expressed as M ± SD. Statistical analysis was carried out with independent-samples t test and the SPSS statistics package (SPSS Inc., Chicago, IL). Differences with p < .05 were considered statistically significant.

### Results

#### Effects of Strenuous Treadmill Running on Blood Parameters and Serum Iron Status in Rats

To determine whether exercise-associated anemia had been induced in rats after the 5 weeks of strenuous treadmill running, the blood parameters were examined. To illuminate the relation of exercise-associated anemia and body iron status, the serum iron and nonheme iron in bone marrow were also examined. The body weight of EG decreased dramatically compared with CG after 5 weeks of strenuous treadmill running. The weight loss is an index of overtraining; the wet weight of gastrocnemius was especially decreased in EG compared with CG (CG: 2.56 ± 0.08 g, EG: 2.23 ± 0.11 g; n = 6; p < .05, Table 2).

The γb, RBC, and Hct levels were significantly lower in EG than in CG (all p < .01, Table 2). The results indicated that the model to produce exercise-associated anemia in rats was successful. Compared with CG, the iron and ferritin in serum of EG were decreased, and nonheme iron in bone marrow and in liver was decreased dramatically (p < .01, p < .05; Table 2). The level of IL-6 and WBC counts are two main indexes of body inflammation. The IL-6 and WBC of EG were strikingly higher than those of CG (all p < .01, Table 2). The data indicated that 5 weeks of strenuous treadmill running induced exercise-associated anemia and body-iron deficiency in rats, which was characterized by reduced amounts of transportable iron (in serum) and preservation (nonheme iron in bone marrow and liver). The increased IL-6 and WBC demonstrated that inflammation was stimulated after 5 weeks of strenuous treadmill running in rats.

#### Expression of DMT1, HCP1, and FPN1 in Rat Duodenal Epithelium

To determine whether iron absorption changed after the 5 weeks of strenuous treadmill running, the iron-absorption proteins DMT1, HCP1, and FPN1 in duodenal epithelium were examined by Western blot. DMT1 is identified as the apical iron-uptake system of enterocytes. DMT1 generates four alternatively spliced mRNAs that differ at their combination of alternative 5′ and 3′ exons (1A or 1B, iron response element [IRE] or non-IRE, separately) and encode four DMT1 polypeptides in different cells and tissues (Hubert & Hentze, 2002). Here, we examined the two proteins’ difference at 3′ untranslated region by the presence or absence of DMT1 (IRE), and DMT1 (non-IRE). It has been reported that the two DMT1 splicing forms are similar in distribution and function. DMT1 pumps nonheme iron from the intestinal lumen into the enterocytes across the apical membrane (Gunshin et al., 1997). HCP1 is an intestinal heme transporter (Shayeghi et al., 2005). FPN1 is a transmembrane protein that is expressed on the basolateral surface of mature duodenal enterocytes and in macrophages of the spleen and liver. FPN1 may play an important role in iron export (Donovan et al., 2000). The results revealed that compared

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Size (bp)</th>
</tr>
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<tbody>
<tr>
<td>Hepcidin</td>
<td>GAAGGCAAGATGGCACAAGCA</td>
<td>TCTCGTCTTGGCCAGATAG</td>
<td>102</td>
</tr>
<tr>
<td>Hemojuvelin</td>
<td>TTCCAATCCTGCCTTTTGAT</td>
<td>GAAAAAGTGCAAGTTCCTCAA</td>
<td>121</td>
</tr>
<tr>
<td>β-actin</td>
<td>GAAATCGTGCGATCCCAAGAG</td>
<td>GCGGCAGTGCCATCTC</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 1 Primer Sequences Used for Quantitative Real-Time Polymerase-Chain-Reaction Analysis
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Table 2  Effects of Strenuous Treadmill Running on Blood Parameters, Serum Iron Status, and Inflammation Index in Rats, M ± SD, n = 6

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Exercise group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>365.00 ± 29.76</td>
<td>295.00 ± 28.11**</td>
</tr>
<tr>
<td>Wet weight of gastrocnemius (g)</td>
<td>2.56 ± 0.08</td>
<td>2.23 ± 0.11*</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>152.56 ± 5.46</td>
<td>103.67 ± 14.49**</td>
</tr>
<tr>
<td>Red blood cells (1012/L)</td>
<td>7.07 ± 0.37</td>
<td>4.78 ± 0.70**</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>45.11 ± 3.18</td>
<td>35.50 ± 4.10**</td>
</tr>
<tr>
<td>Serum iron (mg/L)</td>
<td>1.76 ± 0.32</td>
<td>1.40 ± 0.08*</td>
</tr>
<tr>
<td>Serum ferritin (ng/ml)</td>
<td>42.24 ± 1.33</td>
<td>25.86 ± 2.06**</td>
</tr>
<tr>
<td>Nonheme iron in bone marrow (μg/g dry weight)</td>
<td>1,226.33 ± 22.70</td>
<td>669.31 ± 68.06**</td>
</tr>
<tr>
<td>Nonheme iron in liver (μg/g dry weight)</td>
<td>947.35 ± 21.28</td>
<td>783.14 ± 39.52*</td>
</tr>
<tr>
<td>Interleukin-6 (pg/ml)</td>
<td>21.41 ± 4.42</td>
<td>32.84 ± 7.23**</td>
</tr>
<tr>
<td>White blood cells (109/L)</td>
<td>4.81 ± 0.50</td>
<td>6.95 ± 0.64**</td>
</tr>
</tbody>
</table>

*p < .05 vs. control. **p < .01 vs. control.

with CG, the expression of DMT1 (IRE), DMT1 (non-IRE), and HCP1 of duodenal epithelium in rats with exercise-associated anemia was lower in EG (p < .05; Figure 1). The expression of FPN1 was also significantly lower than that of CG (p < .01; Figure 1). The decreased iron-absorption proteins indicated that the amount of iron absorption might also be reduced in duodenal epithelium in rats with exercise-associated anemia.

Expression of Hepatic Hepcidin mRNA and HJV mRNA in Rat Gastrocnemius

It has been proposed that hepcidin is a negative central regulator of intestinal iron absorption, which is a key regulator in iron homeostasis (Gardenghi et al., 2007). To investigate the regulatory mechanism of DMT1, HCP1, and FPN1 expression in duodenal epithelium, the transcription of hepatic hepcidin mRNA was assayed in rats that had been trained with 5 weeks of strenuous treadmill running. The hepatic hepcidin mRNA was higher in EG than in CG (p < .01; Figure 2). HJV regulates hepcidin expression, and there was higher expression in skeletal muscles. Compared with CG, the expression of HJV mRNA of gastrocnemius in EG was increased (p < .05; Figure 3).

Discussion

The prevalence of iron-deficiency anemia is likely to be higher in athletic populations and groups than in healthy sedentary individuals. In anemic individuals, iron deficiency not only decreases athletic performance but also impairs immune function and leads to other physiologic dysfunction. Here, we found that Hb and Hct were remarkably decreased in rats that experienced 5 weeks of strenuous treadmill running and had exercise-induced anemia. The low level of nonheme iron in rat bone marrow in EG indicated that the iron preservation in the body might have decreased. As we know, iron participates in the synthesis of Hb, and insufficient iron preservation in EG can cause the reduction of Hb. However, the oxidative phosphorylation capacity in the mitochondria is associated with oxygen transported by Hb, and some mitochondrial enzymes and cytochromes are heme-containing proteins. The lower iron status in EG might have caused the decrease in ATP production in mitochondria. It has been well established that reduced Hb concentration and iron deficiency negatively affect the working capacity of skeletal muscle. The decreased serum ferritin showed that body iron stores were decreased in rats with exercise-associated anemia. In addition, serum ferritin is an acute-phase protein whose level is increased during inflammation. However, in this study, serum ferritin was lower in the exercised rats. The reasons might be that serum ferritin is an acute-phase protein. In our experiments, the rats were sacrificed 36 hr after the last exercise session.

The iron losses were increased during exercises, such as gastrointestinal bleeding, hematuria, sweating, and hemolysis, which might be one main reason for exercise-associated anemia (Peeling et al., 2008; Peeling et al., 2009a, 2009b). In addition, the decrease in iron absorption may be another main reason for exercise-associated anemia. Recently, much progress has been made in characterizing the mechanism of duodenal iron absorption. Iron absorption normally occurs in the duodenum and upper part of the jejunum. Generally, the body’s iron status plays an important role in regulating iron absorption in accordance with the body’s needs; absorption increases when iron preservation is lower than normal and decreases when iron levels are high. However, it has also been pointed out that the regulatory ability of iron absorption in the body of an athlete is poor. The iron-absorption rate in the body of a long-distance runner was 16.4%, whereas the iron-absorption rate in
Figure 1 — Effects of strenuous treadmill running on the expression of iron-absorption proteins of duodenal epithelium in rats using Western blot. Immunoreactive proteins were detected using enhanced chemiluminescence and quantified by transmittance densitometry using volume integration with Gel-Pro Analyzer Analysis software to determine the enrichment of the DMT1, HCP1, and FPN1 proteins in rat duodenum. Duodenal epithelium homogenates were from the control group (CG) and exercise group (EG). The expected molecular weight of DMT1 ~56 KDa, FPN1 ~60 KDa, HCP1 ~55 KDa, and β-actin ~42 KDa bands appeared on gels. Data are presented as a percent relative to β-actin (M ± SD, n = 6). *Significant difference by independent-samples t test, p < .05 versus CG. **p < .01.

Figure 2 — Real-time polymerase chain reaction analysis of rat hepatic hepcidin mRNA after 5 weeks of strenuous exercise. Livers were obtained from the control group (CG) and exercise group (EG). Values represent the amount of target mRNA compared with β-actin (M ± SD, n = 6). *Significant difference by independent-samples t test, **p < .01 versus CG.

Figure 3 — Real-time polymerase chain reaction analysis of rat gastrocnemius hemouvelin mRNA after 5 weeks of strenuous exercise. Gastrocnemius was obtained from the control group (CG) and exercise group (EG). Values represent the amount of target mRNA compared with β-actin (M ± SD, n = 6). *Significant difference by independent-samples t test, p < .05 versus CG.
the body of a nonathlete was 30%, which suggests that certain factors regulate iron absorption during exercise (John & Brian, 2000).

Our results indicate that the expression of iron-absorption proteins such as DMT1, HCP1, and FPN1 decreased in strenuously exercised rats compared with those in CG. Therefore, iron absorption could be reduced after high-intensity exercise. As a result, there was not enough iron for the synthesis of Hb in strenuously exercised rats, which further induced exercise-associated anemia. The decreased nonheme iron in bone marrow and liver in strenuously exercised rats provides direct evidence of decreased iron storage. It is possible that the increased hepcidin led to lower expression of iron-absorption proteins in the rats with exercise-associated anemia.

Recent studies have identified hepcidin as a negative regulator of iron absorption in the duodenum (Gardenghi et al., 2007). Hepcidin levels that are inappropriately low or high result in iron overload or iron deficiency, respectively. Serum hepcidin was negatively associated with iron absorption from supplemental and food-based non-heme-iron sources in healthy iron-replete women (Young et al., 2009). Hepatic hepcidin mRNA increased in our rats after the strenuous treadmill exercise. Hepcidin could weaken the functional activity of FPN1 by directly binding to it and causing it to be internalized from the cell surface and degraded, thereby blocking cellular iron efflux (Nemeth et al., 2004). We detected decreased expression of FPN1 at the intestinal duodenum of EG compared with CG and declined iron efflux. To maintain iron homeostasis in enterocytes, the expression of iron-uptake proteins DMT1 and HCP1 was decreased, which ultimately blocked the release of iron from enterocytes in addition to inhibiting the absorption of iron from the gut. The decreased iron absorption during exercise causes an inability to meet the need to synthesize Hb or other mitochondrial enzymes, so the Hb in EG strikingly decreased. Results from other laboratories have also shown increased hepcidin after exercise. Roecker, Meier-Buttermilch, Brechtel, Nemeth, and Ganz’s (2005) data supported the idea that hepcidin increases in female athletes after a marathon; they proposed that the frequently observed iron deficiency in female runners is caused by elevated hepcidin levels. In contrast, more recent studies have reported that daily regulation of serum and urinary hepcidin was not influenced by submaximal cycling exercise in humans with normal iron metabolism (Troade et al., 2009). The different results may be a result of various exercise intensities and body-iron statuses and inconsistent training level. IL-6 and WBC are two main indexes of body inflammation. In our data, IL-6 activity and WBC counts in EG were higher than those in CG, which indicated that inflammation had been induced after 5 weeks of strenuous exercise. Recently, it was reported that increased hepcidin activity may result from exercise-induced inflammation or hemolysis (Collins, Wessling-Resnick, & Knutson, 2008; Peeling et al. 2009a). It is likely that hepcidin provides a molecular link among inflammation, anemia, and iron metabolism (Fleming, 2008; Kemna, Pickkers, Nemeth, van der Hoeven, & Swinkels, 2005; Malyszko & Mysliwiec, 2007). The stimulated inflammation recorded in our exercise-associated-anemia group provides an explanation for the up-regulation of hepcidin activity. Meanwhile, other research has shown a mild increase in serum hepcidin and IL-6 concentrations that impaired iron incorporation in Hb (de Mast et al., 2009; Theurl et al., 2008). Wrighting and Andrews’s (2006) research showed that IL-6 directly regulates hepcidin through induction and subsequent promoter binding of signal transducer and activator of transcription 3. Transcription 3 is necessary and sufficient for IL-6’s responsiveness to the hepcidin promoter. Besides inflammation, hepcidin expression is also influenced by systemic stimuli such as iron stores, the rate of erythropoiesis, hypoxia, and oxidative stress (Darshan & Anderson, 2009). Recent research showed that HJV has a key role in hepcidin regulation (Silvestri, Pagani, & Camaschella, 2008). HJV is related to the hypoxic response. The highest expression of HJV is found in skeletal muscle. The recent finding that HJV is released from skeletal muscle in iron deficiency suggests that this tissue may play a critical role in iron homeostasis. Our results showed that the HJV mRNA of skeletal muscle increased. However, it is still not clear whether there are other cooperators that affect hepcidin in exercise-associated anemia. Further studies are needed to characterize the effects of erythropoiesis, hypoxia, and oxidative stress on hepcidin and other iron-related regulators during different-intensity exercises.

**Conclusions**

Taken together, our findings provide a mechanism through which exercise-associated anemia can be regulated by hepcidin. Our data suggest that inflammation was stimulated after 5 weeks of strenuous exercise, which resulted in increased hepcidin expression. As a negative regulatory signal, hepcidin could combine with FPN1, and consequently the expressions of iron absorption proteins decline. In turn, transport and iron preservation decreased. Therefore, there was not enough iron to synthesize Hb and RBC. As a result, exercise-associated anemia was induced. Iron homeostasis was severely affected by high-intensity exercise in rats. It should be noted that our current understanding of strenuous-exercise-mediated regulation of iron metabolism is still incomplete. Additional studies will be necessary to explain the mechanism of hepcidin’s effect on iron transport and utilization during exercise.

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References


