Short Communication

Hepcidin deficiency undermines bone load-bearing capacity through inducing iron overload

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A R T I C L E   I N F O

Article history:
Received 5 December 2013
Received in revised form 24 January 2014
Accepted 4 February 2014
Available online 19 February 2014

Keywords:
Hepcidin deficiency
Iron overload
Bone resorption
Bone strength

A B S T R A C T

Osteoporosis is one of the leading disorders among aged people. Bone loss results from a number of physiological alterations, such as estrogen decline and aging. Meanwhile, iron overload has been recognized as a risk factor for bone loss. Systemic iron homeostasis is fundamentally governed by the hepcidin–ferroportin regulatory axis, where hepcidin is the key regulator. Hepcidin deficiency could induce a few disorders, of which iron overload is the most representative phenotype. However, there was little investigation of the effects of hepcidin deficiency on bone metabolism. To this end, hepcidin-deficient (Hamp1−/−) mice were employed to address this issue. Our results revealed that significant iron overload was induced in Hamp1−/− mice. Importantly, significant decreases of maximal loading and maximal bending stress were found in Hamp1−/− mice relative to wildtype (WT) mice. Moreover, the levels of the C-telopeptide of type I collagen (CTX-1) increased in Hamp1−/− mice. Therefore, hepcidin deficiency resulted in a marked reduction of bone load-bearing capacity likely through enhancing bone resorption, suggesting a direct correlation between hepcidin deficiency and bone loss. Targeting hepcidin or the pathway it modulates may thus represent a therapeutic for osteopenia or osteoporosis.

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1. Introduction

According to the data of the World Health Organization, osteoporosis has become a global issue and an increasing threat to human health (Alexandre, 1995). Osteoporosis is a common disease characterized by low bone mineral mass, associated with a high incidence of bone fractures (Cooper and Aihie, 1995; Guggenbuhl et al., 2005). Bone metabolism is maintained through a kinetic balance between formation and resorption. Bone formation is performed by osteoblasts, while bone resorption is carried out by osteoclasts (Fohr et al., 2003; Mizuno et al., 1998; Suda et al., 1996). Disruption of this balance is likely associated with bone loss (Suda et al., 1996). There are a number of factors that may account for osteopenia or osteoporosis, such as estrogen deficiency and abnormal calcium metabolism (Chevalley et al., 1994; Kanis, 1994). Additionally, Delbarre and his colleagues reported that osteoporosis is a complication of hereditary hemochromatosis (HH) half a century ago (Delbarre, 1964). Since then, a number of clinical and experimental studies have documented that iron overload represents a risk factor responsible for bone loss, especially in patients suffering from HH (Guggenbuhl et al., 2011a), African hemosiderosis (Lorincz et al., 1974), thalassemia (Salama et al., 2006), sickle cell disease (Adams-Graves et al., 2013), and liver diseases (Diamond et al., 1990). A recent study described that 25% of HH patients were diagnosed with osteoporosis and 41% developed osteopenia (Valenti et al., 2009). HH is a genetic disorder, associated with iron overload in various organs (Fleming and Sly, 2002). In parallel to this finding, hemochromatosis (HFE)-deficient male mice, a mouse model of HH, developed osteoporosis with low bone mass (Guggenbuhl et al., 2005, 2011b). Meanwhile, numerous clinical studies suggested that patients with osteoporosis accompanied iron accumulation in bone (Jian et al., 2009). Together, iron homeostasis is closely coupled to bone metabolism, and excessive iron accumulation is recognized as a risk factor for osteoporosis (Haidar et al., 2011; Kudo et al., 2008; Tsay et al., 2010; Valenti et al., 2009).

Iron homeostasis is fundamentally governed by the hepcidin–ferroportin regulatory axis (Park et al., 2001). Hepcidin, a 25 amino acid peptide, is the master hormone in modulating systemic iron homeostasis (Ganz, 2011). Its transcription is stimulated by iron, inflammatory cytokines and bone morphogenetic proteins (BMPs), and repressed by signaling from anemia and hypoxia (Ganz, 2011). Hepcidin inhibits iron egress out of cells through binding and inducing degradation of its receptor ferroportin. Ferroportin, the only known

Abbreviations: WT, wildtype; CTX-1, C-telopeptide of type I collagen; HH, hemochromatosis; HFE, hemochromatosis; BMP, bone morphogenetic protein; PBS, phosphate-buffered saline; OD, optical density; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.gene.2014.02.023
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mammalian iron exporter is mainly expressed in epithelial cells in duodenum and macrophages (Jelic et al., 2013; Pinnix et al., 2010). Suppression of hepcidin expression or hepcidin deficiency would cause enhanced iron egress out of macrophages, leading to iron overload in various organs, associated with neoplasia, arthropathy and neurodegenerative diseases (Jelic et al., 2013; Pinnix et al., 2010).

However, there was still no investigation of the effects of hepcidin deficiency on bone metabolism. To this end, we used the hepcidin-deficient (hepcidin knockout, Hamp1−/−) mice to study bone metabolism under the setting of disordered iron homeostasis. We overall demonstrated that iron overload due to hepcidin deficiency greatly undermined bone strength and bone load-bearing capacity. Thus, targeting hepcidin or the signaling it mediates may represent a novel therapeutic for osteoporosis.

2. Materials and methods

2.1. Animal experiment

Hamp1−/− mice were originally provided by Dr. Sophie Vaulont (Lesbordes-Brion et al., 2006) and currently with the C57BLK/6 background (Ramos et al., 2012). All mice were housed in the central animal facility of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Male Hamp1−/− mice and wildtype (WT) mice with the same genetic background were sacrificed at 8 weeks, 18 weeks and 30 weeks. Sera and organ specimens were collected. Tibias were removed and packed in gauze soaked with phosphate-buffered saline (PBS), and stored at −80 °C for future analyses.

2.2. Iron parameters

Serum iron concentration was determined with a serum iron detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Hepatic iron content was assessed as previously described (Liu et al., 2007; Yamasaki and Hagiwara, 2009). PBS was used to keep specimens moist during testing.

2.3. Assessments of bone metabolic markers

Serum osteocalcin and C-telopeptide of type I collagen (CTX-1) were assayed by ELISA (Rapidbio; RapidBio, West Hills, CA, USA), according to the manufacturer’s instructions. Briefly, the specimens and standards were added into 96-well plates, and then incubated for 30 min at 37 °C. After washing five times, HRP-conjugate reagent was added, followed by another round of incubation and washing. Optical density (OD) at 450 nm was measured after adding stop solution within 15 min. Finally, serum osteocalcin and CTX-1 concentrations were calculated according to the standard curves.

2.4. Bone biomechanical analyses

Biomechanical properties of tibias were determined by the three-point bending test using a universal material test machine (AG-IS; Shimadzu Co., Kyoto, Japan) at room temperature, as described in previous studies (Mattila et al., 1999; Yamasaki and Hagiwara, 2009). The distance between the supporting rods had a fixed length (L) of 12 mm. Load was applied at a constant deformation rate of 2 mm/min. The deflection of the tibia was loaded until fracture occurred, which provided measurement of yield and fracture parameters. Yield represents the point at which the bone ceases to behave elastically. Data were automatically recorded in a computer interfaced to the testing machine, and a typical load-deformation curve was created. The material properties of bone including the maximal loading and maximal bending stress were calculated according to the established formulas (Mattila et al., 1999; Yamasaki and Hagiwara, 2009). PBS was used to keep specimens moist during testing.

2.5. Statistical analysis

The SPSS Statistics 17.0 package was utilized to analyze the date. One-way analysis of variance (ANOVA) was applied to determine the mean differences among groups compared to the control. The difference between two groups was determined using the independent t-test. Data were shown in mean ± SD. P < 0.05 was considered statistically significant.

3. Results

3.1. Systemic iron overload in hepcidin-deficient mice

To look into iron concentrations of Hamp1−/− mice, we compared serum and tissue iron concentrations between hepcidin-deficient mice and WT mice of different ages. As shown in Fig. 1a, serum iron level was increased by approximately 25% in Hamp1−/− mice relative to WT mice at 8 weeks (p < 0.05). This difference became even greater when mice got older, as evidenced by over 40% and 50% increase of serum iron in Hamp1−/− mice compared to WT mice at 18 weeks and 30 weeks, respectively (Fig. 1a, p < 0.05). Moreover, there was a clear time-dependent increase of serum iron for Hamp1−/− mice from 8 weeks to 30 weeks (Fig. 1a, p < 0.05). However, the serum iron level remained consistent in WT mice over the time course from 8 weeks to 30 weeks (Fig. 1a, p > 0.05). Similarly, liver iron content was greatly increased in Hamp1−/− mice compared to WT mice for all ages tested (Fig. 1b, p < 0.05). There was an increase of liver iron concentrations in a time-dependent manner (Fig. 1b, p < 0.05), and hepatic iron concentrations were similar at different ages (Fig. 1b). Consistent with previous findings (Masaratana et al., 2011), our data revealed that hepcidin deficiency led to remarkable systemic iron overload, as reflected by increased serum iron and hepatic iron.

![Fig. 1. Iron content in WT mice and Hamp1−/− mice at different ages. Serum iron (a) and hepatic iron (b) were shown (n = 4-5).](image-url)
3.2. Hepcidin deficiency undermined bone load-bearing capacity

To investigate the alterations of bone strength devoid of hepcidin, the biomechanical parameters of bone were examined. As shown in Fig. 2a, the maximal loading of tibias was similar between WT mice and Hamp1^{-/-} mice at the age of 8 weeks. The maximal loading was significantly reduced by 20% in Hamp1^{-/-} mice at the age of 18 weeks (p < 0.05); however, it kept consistent for an 18 week old WT mice (p > 0.05). More strikingly, the maximal loading was further reduced approximately by 50% when mice reached 30 week old for Hamp1^{-/-} mice compared to that for mice of 8 week old (Fig. 2a, p < 0.05). Meanwhile, the maximal loading was reduced by 12% only in WT mice when they were 30 week old, and it was still much greater (over 42%) than that for hepcidin-deficient mice (Fig. 2a, p < 0.05). Similar to the changes of the maximal loading, the maximal bending was also declined in Hamp1^{-/-} mice compared to WT mice at the ages of 18 weeks and 30 weeks, especially at 30 weeks (Fig. 2b, p < 0.05). In other words, the maximal bending stress declined more rapidly in hepcidin deficient mice compared to WT mice from 8 weeks to 30 weeks (Fig. 2b, p < 0.05). These results together demonstrated that hepcidin deficiency significantly impaired mechanical properties, especially for aged mice.

3.3. Hepcidin deficiency enhanced bone resorption

To elucidate the mechanism underlying impaired bone strength under hepcidin deficiency, we looked into possible alterations to the delicate balance between bone formation and bone resorption. Osteocalcin is secreted by osteoblasts in their maturation stage in order to sustain mineralization, and, thus, osteocalcin is widely used as a marker to recognize the activity of bone formation (Christenson et al., 2012; Fiore et al., 1984). CTX-1 is a degraded product of type I collagen, and it reflects the activity of osteoclast-conducted bone resorption (Lian et al., 1982). CTX-1 is thus used as a marker indicating osteoclastic activity and bone resorption (Reginster et al., 2001). Regarding serum osteocalcin concentration, it was similar at 8 weeks and 18 weeks for both WT and Hamp1^{-/-} mice, and no significant difference was found between WT and Hamp1^{-/-} mice (Fig. 3a). Serum osteocalcin level was reduced by approximately 39% in both WT and Hamp1^{-/-} mice at 30 weeks compared to that at 8 weeks or 18 weeks (Fig. 3a, p < 0.05). Consistent with previous studies, this finding suggested attenuation of bone formation activity in aged mice. There was only a slight decrease of serum osteocalcin in Hamp1^{-/-} mice compared to that in WT mice at 30 weeks with no significant difference (Fig. 3a, p > 0.05), implying bone formation was not significantly undermined due to hepcidin deficiency. However, we observed a distinct pattern for the CTX-1 concentration changes between WT mice and Hamp1^{-/-} mice during aging. As shown in Fig. 3c, the CTX-1 level was consistent for WT mice at different ages of 8 weeks, 18 weeks and 30 weeks, indicating the activity of bone resorption was not significantly altered during aging. These results were similar to the previous observations that the osteoclastic capability in bone resorption retained at a similar level for aged mice relative to young mice (Hauschka and Carr, 1982; Lian and Gudberg, 1988). Importantly, we found that the CTX-1 concentration was increased by approximately 25% in Hamp1^{-/-} mice compared to WT mice at 8 weeks and 18 weeks (Fig. 3c, p < 0.05). And the CTX-1 concentration was further increased (by 32%) in Hamp1^{-/-} mice in comparison to WT mice at 30 weeks (Fig. 3c, p < 0.05). Moreover, there was a clear time-dependent increased of CTX-1 level in Hamp1^{-/-} mice from 8 weeks to 30 weeks (Fig. 3c, p < 0.05), revealing hepcidin deficiency further instigated bone resorption over the time course. These data together demonstrated that hepcidin deficiency greatly promoted osteoclast-induced bone resorption, especially in old mice, without significantly affecting bone formation.
4. Discussion

Hepcidin plays a central role in maintaining iron homeostasis by suppressing iron absorption from duodenum and iron egress from cells (mainly macrophages and hepatocytes) by inducing degradation of iron exporter ferroportin (Nemeth and Ganz, 2009). Thus, the hepcidin–ferroportin regulatory axis fundamentally governs systematic iron homeostasis, through determining iron uptake, egress and localization (Andrews, 2008; Ganz, 2011; Knutson, 2010). Deregelated hepcidin–ferroportin axis is implicated in a wide spectrum of diseases, including iron diseases (Nemeth and Ganz, 2009), such as HH and anemia of inflammation, and various chronic diseases (e.g. cancers) (Brisso et al., 2011; Ganz et al., 2008; Kamai et al., 2009; Maes et al., 2010; Tanno et al., 2011). Hepcidin deficiency would lead to enforced iron absorption from duodenum and iron export from macrophages, leading to increased iron retention and even iron overload in various organs (Brisso et al., 2011; Ganz et al., 2008; Kamai et al., 2009; Maes et al., 2010; Tanno et al., 2011). In fact, hepcidin-null mice developed severe iron overload soon after weaning, as evidenced by a similar concentration between hepcidin-null mice and WT mice. These results demonstrated that hepcidin deficiency led to enforced iron absorption, and macrophage hepcidin might play a role in iron absorption. Indeed, a recent study supported this statement that excess iron could stimulate bone resorption through TNF-α-dependent osteoclastic activity in iron overload mice (Rong, 2012). In addition to hepatocytes, macrophages were also verified to express hepcidin, and macrophage hepcidin might play an important role in modulating inflammatory responses (Wu et al., 2012). Osteoclasts are a class of specialized macrophages in bone. However, whether hepcidin is also expressed in osteoclasts keeps unknown. Meanwhile, whether the absence of hepcidin activates osteoclasts and then stimulates bone resorptive activity warrants detailed investigation.

To summarize, our results highlighted a crucial role of hepcidin in maintaining bone metabolism. Hepcidin deficiency resulted in enhanced bone resorption and consequential bone loss, associated with impaired bone biomechanical properties, such as a large reduction of the maximal loading and the maximal bending stress. The loss of bone strength in the absence of hepcidin should result, at least mostly, from iron overload for hepcidin-null mice. A proposed schematic depicting the mechanisms underlying hepcidin deficiency-induced bone loss is illustrated in Fig. 4. The detailed molecular mechanisms certainly need further studies.

Conflict of interest

No potential conflicts of interest were disclosed from the authors. There are no non-financial competing interests to declare in relation to this manuscript.

Acknowledgments

This work was supported by a grant under the national “973” program (grant number: 2014CB932000) and the National Natural Science Foundation of China (grant number: 21377159). We thank Dr. Sophie Vaulont and Dr. Tomas Ganz for providing us hepcidin-null mice. We thank the laboratory members for their great assistance with the experiments and reagents.

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