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Hepcidin expression in the liver of rats fed a magnesium-deficient diet

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Abstract

Mg deficiency accelerates Fe accumulation in the liver, which may induce various metabolic disturbances. In the present study, we examined the gene expression of *Hepcidin*, a peptide hormone produced in the liver to regulate intestinal Fe absorption negatively, in Mg-deficient rats. Although liver Fe concentration was significantly higher in rats fed an Mg-deficient diet for 4 weeks than in rats fed a control diet, *Hepcidin* expression in the liver was comparable between the dietary groups. Previous studies revealed that Fe overload up-regulated *Hepcidin* expression through transcriptional activation by Fe-induced bone morphogenetic protein (Bmp) 6, a growth/differentiation factor belonging to the transforming growth factor-β family, in the liver. Mg deficiency up-regulated the expression of *Bmp6* but did not affect the expression of inhibition of DNA binding 1, a sensitive Bmp-responsive gene. In addition, the expression of Bmp receptors such as activin receptor-like kinase 2 (*Alk2*), activin receptor type IIA (*Actr2a*), activin receptor type IIB (*Actr2b*) and Bmp type II receptor (*Bmpr2*) was lower in the liver of Mg-deficient rats than in that of control rats. The present study indicates that accumulation of hepatic Fe by Mg deficiency is a stimulant inducing *Bmp6* expression but not *Hepcidin* expression by blunting Bmp signalling possibly resulting from down-regulation of the receptor expression. Unresponsive Hepcidin expression may have a role in Mg deficiency-induced changes related to increased liver Fe.

Key words: Magnesium deficiency: Hepcidin: Liver iron content: Bone morphogenetic protein

Mg is a cofactor of numerous enzymes and plays an essential role in a wide range of fundamental cellular reactions. Insufficient Mg intake therefore induces numerous abnormalities in rodents[1]. Mg deficiency induced oxidative stress, which was evaluated by lipid peroxidation, and apoptosis in rat liver[2,3]. In addition, TAG and total cholesterol concentrations were increased in the liver and serum of Mg-deficient rats[4]. These features resemble the altered metabolism in the liver of rats fed a high-Fe diet; Fe overload enhanced lipid peroxidation, increased apoptotic cell number and elevated liver fat concentration and serum lipid concentrations, including TAG and total cholesterol[5–8]. In view of the accumulation of hepatic Fe in Mg-deficient rats[2,9,10], increased hepatic Fe content may cause various Mg-deficiency-related abnormalities in the liver.

Hepcidin was originally isolated from human urine as an anti-microbial peptide[11] and is currently recognised as a hormone secreted from the liver in response to the Fe overload; it negatively regulates intestinal Fe absorption through internalisation and degradation of an Fe transporter, ferroportin[12]. Considering that hepatic *Hepcidin* transcription is triggered by excess Fe[13,14], Mg deficiency is expected to increase *Hepcidin* expression in the liver; however, a previous study revealed an increase in the intestinal absorption of Fe in Mg-deficient rats[10], suggesting the failure of regulatory Fe metabolism by Hepcidin. The present study examined the expression of hepatic *Hepcidin* in Mg-deficient rats.

Materials and methods

Animals and diets

A total of twelve 5-week-old male Sprague–Dawley rats were purchased from SLC Japan (Shizuoka, Japan) and cared for according to the Guide for the Care and Use of Laboratory Animals (Animal Care Committee, Kyoto University, Kyoto, Japan). They were individually housed in stainless-steel cages in a temperature-, humidity- and light-controlled room (24°C, 60 %, 12 h light–12 h dark cycle). All rats were fed a control diet (American Institute of Nutrition-93G diet)[15] for a 5 d adaptation period, followed by feeding either the control diet or an Mg-deficient diet (American Institute of Nutrition-93G-based diet with Mg-free mineral mixture). The Mg content

Abbreviations: Actr2, activin receptor type II; Alk, activin receptor-like kinase; Bmp, bone morphogenetic protein; *Bmpr2*, bone morphogenetic protein type II receptor; Hfe, haemochromatosis; Id1, inhibition of DNA binding 1; Tfr, transferrin receptor.

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determined in the control diet and the Mg-deficient diet was 49·6 and 4·2 mg/100 g, respectively. Rats were pair-fed their respective experimental diets and were allowed free access to demineralised water for 4 weeks. After the feeding trial, the rats were killed by collecting blood from the abdominal aorta under isoflurane anaesthesia, and the liver was collected.

Measurement of dietary magnesium and calcium, serum magnesium, liver iron and liver thiobarbituric acid-reactive substances

Dietary sample, and serum and liver samples were digested with trace element-grade HNO_3 and H_2O_2 (Wako, Osaka, Japan), and dietary and serum Mg and liver Fe were determined by atomic absorption spectrophotometry (AA-6600F; Shimadzu, Kyoto, Japan). Analytical accuracy of liver Fe was confirmed by analysis of a certified reference material of bovine liver (Standard Reference Material 1577b; National Institute of Standards and Technology, Gaithersburg, MD, USA). The liver samples were also homogenised in chilled saline by Polytron (PT1600E; Kinematica, Lucerne, Switzerland), and the homogenate was centrifuged at 105 000 ***g*** for 30 min at 4°C. The concentration of thiobarbituric acid-reactive substances in the supernatant was determined using a commercial kit (OXI-TEK TBARS Assay Kit; ZeptoMetrix, Buffalo, NY, USA) according to the manufacturer's instructions.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from the liver samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Absorbance at 260 nm was measured to quantify RNA concentration, and simultaneously the ratio of absorbance at 260 nm to that at 280 nm was monitored to assess the purity of RNA. Quantitative RT-PCR was carried out as described previously[16,17]. The following oligonucleotides were used as PCR primers: 5′-gggcagaaagcaagactgat-3′ and 5′-ttacagcatttacagcagaagagg-3′ for *Hepcidin* (GenBank accession no. NM_053469.1); 5′-gacagcagagtcgcaatcg-3′ and 5′-agctcacgtaaagctcatgc-3′ for bone morphogenetic protein (*Bmp*) 6 (GenBank accession no. NM_013107); 5′-gcgagatcagtgccttgg-3′ and 5′-ttttcctcttgcctcctgaa-3′ for the inhibition of DNA binding 1 (*Id1*) (GenBank accession no. NM_012797.2); 5′-actacctgcagagggactgc-3′ and 5′-actttcaccaaagtaggcacttg-3′ for haemochromatosis (*Hfe,* GenBank accession no. NM_053301.4); 5′-gtagcatcgggagccaac-3′ and 5′-tcaaaggctgcaggaagatt-3′ for *Hemojuvelin* (GenBank accession no. NM_001012080.1); 5′-gagttcactgacatcatcaagca-3′ and 5′-tccagcctcacgaggagtat-3′ for transferrin receptor 1 (*Tfr1*) (GenBank accession no. NM_022712); 5′-tcagtaacatctttgcgtgcat-3′ and 5′-gccccgataacgacatagtg-3′ for *Tfr2* (GenBank accession no. NM_001105916). PCR primers for activin receptor-like kinase 2 (*Alk2*), activin receptor-like kinase 3 (*Alk3*), activin receptor type IIA (*Actr2a*), activin receptor type IIB (*Actr2b*), Bmp type II receptor (*Bmpr2*) and glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*) were described previously [18]. The relative mRNA level is expressed as a ratio of the *G3pdh* mRNA level.

Statistical analyses

Data are expressed as means with their standard errors. Differences between the treatments were examined by Student's *t* test. Differences of $P<0·05$ were considered significant.

Results and discussion

Consistent with the previous results[2,9,10], the serum concentration of Mg was significantly lower in rats fed the Mg-deficient diet (Table 1). In addition, liver concentrations of Fe and thiobarbituric acid-reactive substances, an index of oxidative stress, were higher in the Mg-deficient group. Expression of hepatic *Tfr1* was significantly lower in Mg-deficient rats than in control rats, whereas that of hepatic *Tfr2* was comparable between the groups. These results were consistent with the results of Fe-overloaded mice[19,20]. Fe-responsive elements within the untranslated region are present for *Tfr1* but not for *Tfr2* mRNA, which explains why the mRNA level of *Tfr1* but not *Tfr2* was negatively regulated by Fe status[21]. Thus, effects of Mg deficiency on the expression of *Tfr1* and *Tfr2* could reflect Fe status in the liver.

Mg deficiency did not affect the gene transcript level of *Hepcidin* in the liver (Table 2). Hepcidin is a hormone that regulates intestinal Fe absorption negatively[12]. *Hepcidin* expression is transcriptionally induced in response to the elevation of hepatic Fe[12]. The present study revealed that the expression of *Hepcidin* in the liver is not up-regulated by Mg deficiency, irrespective of the enhanced accumulation of hepatic Fe. Thus, it is suggested that the lack of response of the *Hepcidin* expression is at least partly responsible for Mg-deficiency-induced dysregulation of Fe homeostasis.

Expression of *Bmp6* was significantly higher in Mg-deficient rats than in control rats, but *Id1* expression was not different between the dietary groups (Table 2). In the liver, *Hepcidin* is transcriptionally regulated by Bmp6[22,23], and *Id1* is a representative Bmp-responsive gene regulated at the transcription level[24]. Previous studies revealed that Fe overload up-regulated the expression of *Bmp6* and *Id1* in the liver[14,25]. Exogenous Bmp6 increased *Hepcidin* expression in Hep3B cells[22] as well as in the liver[23]. Furthermore, targeted disruption of the *Bmp6* gene decreased the expression of

Table 1. Effect of magnesium deficiency on the serum concentration of magnesium, liver concentration of iron and thiobarbituric acid-reactive substances (TBARS), and hepatic expression of iron-related molecules (Mean values with their standard errors, *n* 6)

	Control		Mg deficiency	
	Mean	SEM	Mean	SEM
Serum Mg (mg/l)	22·1	1·7	7·3**	1·2
Liver Fe (μg/g)	87·8	5·8	148·1**	14·9
Liver TBARS (nmol/g)	35·9	2·4	57·8**	1·7
Fe-related molecules				
Tfr1	1·00	0·18	0·45*	0·11
Tfr2	1·00	0·04	0·93	0·06

Tfr, transferrin receptor.
Mean values were significantly different from those of the control group: * $P<0·05$, ** $P<0·01$.

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Table 2. Effect of magnesium deficiency on the hepatic expression of *Hepcidin*, bone morphogenetic protein (*Bmp*) 6, inhibition of DNA binding 1 (*Id1*), haemochromatosis (*Hfe*), *Hemojuvelin* and Bmp receptors

(Mean values with their standard errors, *n* 6)

	Control		Mg deficiency	
	Mean	SEM	Mean	SEM
Hepcidin	1·00	0·13	0·98	0·11
Bmp6	1·00	0·29	2·22*	0·38
Id1	1·00	0·41	1·57	0·72
Hfe	1·00	0·05	0·70**	0·06
Hemojuvelin	1·00	0·21	1·66**	0·17
Bmp receptors				
Type I receptors				
Alk2	1·00	0·15	0·44**	0·06
Alk3	1·00	0·10	0·70	0·10
Type II receptors				
Actr2a	1·00	0·09	0·55**	0·06
Actr2b	1·00	0·08	0·65*	0·09
Bmpr2	1·00	0·13	0·51*	0·04

Alk, activin receptor-like kinase; Actr2, activin receptor type II; Bmpr2, Bmp type II receptor.
Mean values were significantly different from those of the control group: * $P<0·05$, ** $P<0·01$.

Hepcidin and accumulated Fe in the liver[23,26]. Thus, Bmp6 is a signal mediator linking Fe accumulation and *Hepcidin* expression, although transcriptional activation of the *Bmp6* gene by excess Fe accumulation is currently unclear at the molecular level[27]. In the present study, the expression of *Bmp6* was increased 2·2-fold in rats fed the Mg-deficient diet. The extent of the response was comparable with a previous result; feeding a high-Fe diet for 7 weeks resulted in a 1·8-fold increase in *Bmp6* expression and sevenfold increase in *Hepcidin* expression in DBA/2 mice[14]. Mg deficiency may blunt the Bmp pathway by altering the function of factors involved in hepatic *Hepcidin* induction.

The gene transcript level of *Hfe* was significantly lower in Mg-deficient rats than in control rats, whereas that of *Hemojuvelin* was higher in Mg-deficient rats (Table 2). Upon Bmp binding to the two types of receptors, i.e. type I and type II serine/threonine receptors, the receptor complex phosphorylates and activates Smad1/5/8, leading to transcriptional activation of the target genes such as *Id1*[28]. The strength and duration of the Bmp signal are regulated at multiple steps; expression of co-receptors for Bmp is involved in the fine-tuning of Bmp signalling[28]. Previous studies revealed that Hemojuvelin, which is a gene product of *Hfe2* and a co-receptor of Bmp, including Bmp6, enhances *Hepcidin* expression both *in vitro* and *in vivo*[22,29,30]. In view of the up-regulation of *Hemojuvelin* expression in Mg-deficient rats, the co-receptor is unlikely to be involved in the unresponsiveness to Bmp6.

Recently, Kautz *et al.*[25] revealed that the expression of *Bmp6* was enhanced in Hfe-null mice, but hepatic Bmp signalling, such as phosphorylation of Smad1/5/8 and *Id1* expression, was not accelerated. Similar results were also recently obtained in patients with hereditary haemochromatosis with mutation of the *HFE* gene[31]. In the liver of Fe-overloaded mice, both *Hfe* and *Hemojuvelin* expressions were increased[20]. Therefore, the blunting of Bmp signalling at the gene transcript level of *Hepcidin* may be explained by the result that Mg deficiency down-regulated *Hfe* expression in the liver, although up-regulation of *Hepcidin* expression in response to Bmp2, Bmp4 and Bmp9 in primary hepatocytes from wild-type mice was comparable with those from Hfe-null mice[32].

Down-regulation of the expression of Bmp receptors is possibly related to blunting of Bmp signalling in Mg-deficient rats. Among Bmp receptors, expression of hepatic *Alk2*, *Actr2a*, *Actr2b* and *Bmpr2* was significantly lower in Mg-deficient rats than in control rats (Table 2); expression of activin receptor-like kinase 6 (*Alk6*), a Bmp type I receptor, was not significant (data not shown). Receptor expression level also determines the strength of Bmp signalling[28,33].

In conclusion, the accumulation of hepatic Fe by Mg deficiency is a stimulant inducing *Bmp6* expression but not *Hepcidin* expression by blunting Bmp signalling possibly resulting from down-regulation of the receptor expression. Unresponsive Hepcidin expression may have a role in Mg-deficiency-induced changes related to increased liver Fe.

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References

1. Mazur A, Maier JA, Rock E, *et al.* (2007) Magnesium and the inflammatory response: potential physiopathological implications. *Arch Biochem Biophys* **458**, 48–56.
2. Vormann J, Günther T, Höllriegl V, *et al.* (1995) Effect of various degree and duration of magnesium deficiency on lipid peroxidation and mineral metabolism in rats. *Nutr Biochem* **6**, 681–688.
3. Martin H, Uring-Lambert B, Adrian M, *et al.* (2008) Effects of long-term dietary intake of magnesium on oxidative stress, apoptosis and ageing in rat liver. *Magnes Res* **21**, 124–130.
4. Akiyama S, Uehara M, Katsumata S, *et al.* (2008) Effects of dietary ascorbic acid supplementation on lipid peroxidation and the lipid content in the liver and serum of magnesium-deficient rats. *Magnes Res* **21**, 232–236.
5. Wang GS, Eriksson LC, Xia L, *et al.* (1999) Dietary iron overload inhibits carbon tetrachloride-induced promotion in chemical hepatocarcinogenesis: effects on cell proliferation, apoptosis, and antioxidation. *J Hepatol* **30**, 689–698.
6. Fischer JG, Glauert HP, Yin T, *et al.* (2002) Moderate iron overload enhances lipid peroxidation in livers of rats, but does not affect NF-κB activation induced by the peroxisome proliferator, Wy-14,643. *J Nutr* **132**, 2525–2531.
7. Turbino-Ribeiro SM, Silva ME, Chianca DA Jr, *et al.* (2003) Iron overload in hypercholesterolemic rats affects iron homeostasis and serum lipids but not blood pressure. *J Nutr* **133**, 15–20.

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8. Silva M, Silva ME, de Paula H, *et al.* (2008) Iron overload alters glucose homeostasis, causes liver steatosis, and increases serum triacylglycerols in rats. *Nutr Res* **28**, 391–398.
9. Kimura M & Yokoi K (1996) Iron accumulation in tissues of magnesium-deficient rats with dietary iron overload. *Biol Trace Elem Res* **51**, 177–197.
10. Sanchez-Morito N, Planells E, Aranda P, *et al.* (2000) Influence of magnesium deficiency on the bioavailability and tissue distribution of iron in the rat. *J Nutr Biochem* **11**, 103–108.
11. Park CH, Valore EV, Waring AJ, *et al.* (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* **276**, 7806–7810.
12. Lee PL & Beutler E (2009) Regulation of hepcidin and iron-overload disease. *Annu Rev Pathol* **4**, 489–515.
13. Pigeon C, Ilyin G, Courselaud B, *et al.* (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* **276**, 7811–7819.
14. Kautz L, Meynard D, Monnier A, *et al.* (2008) Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. *Blood* **112**, 1503–1509.
15. Reeves PG (1997) Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr* **127**, 838S–841S.
16. Furutani Y, Murakami M & Funaba M (2009) Differential responses to oxidative stress and calcium influx on expression of the transforming growth factor-β family in myoblasts and myotubes. *Cell Biochem Funct* **27**, 578–582.
17. Suenaga M, Matsui T & Funaba M (2010) BMP inhibition with dorsomorphin limits adipogenic potential of preadipocytes. *J Vet Med Sci* **72**, 373–377.
18. Nishino Y, Ooishi R, Kurokawa S, *et al.* (2009) Gene expression of the TGF-β family in rat brain infected with Borna disease virus. *Microbes Infect* **11**, 737–743.
19. Fleming RE, Migas MC, Holden CC, *et al.* (2000) Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. *Proc Natl Acad Sci U S A* **97**, 2214–2219.
20. Theurl I, Ludwiczek S, Eller P, *et al.* (2005) Pathways for the regulation of body iron homeostasis in response to experimental iron overload. *J Hepatol* **43**, 711–719.
21. Trinder D & Baker E (2003) Transferrin receptor 2: a new molecule in iron metabolism. *Int J Biochem Cell Biol* **35**, 292–296.
22. Babitt JL, Huang FW, Xia Y, *et al.* (2007) Modulation of bone morphogenetic protein signaling *in vivo* regulates systemic iron balance. *J Clin Invest* **117**, 1933–1939.
23. Andriopoulos B Jr, Corradini E, Xia Y, *et al.* (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet* **41**, 482–487.
24. Korchynskyi O & ten Dijke P (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* **277**, 4883–4891.
25. Kautz L, Meynard D, Besson-Fournier C, *et al.* (2009) BMP/Smad signaling is not enhanced in Hfe-deficient mice despite increased Bmp6 expression. *Blood* **114**, 2515–2520.
26. Meynard D, Kautz L, Darnaud V, *et al.* (2009) Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet* **41**, 478–481.
27. Camaschella C (2009) BMP6 orchestrates iron metabolism. *Nat Genet* **41**, 386–388.
28. Miyazono K, Kamiya Y & Morikawa M (2010) Bone morphogenetic protein receptors and signal transduction. *J Biochem* **147**, 35–51.
29. Babitt JL, Huang FW, Wrighting DM, *et al.* (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet* **38**, 531–539.
30. Xia Y, Babitt JL, Sidis Y, *et al.* (2008) Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin. *Blood* **111**, 5195–5204.
31. Ryan JD, Ryan E, Fabre A, *et al.* (2010) Defective bone morphogenic protein signaling underlies hepcidin deficiency in HFE hereditary hemochromatosis. *Hepatology* **52**, 1266–1273.
32. Truksa J, Peng H, Lee P, *et al.* (2006) Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. *Proc Natl Acad Sci U S A* **103**, 10289–10293.
33. Murakami M, Kawachi H, Ogawa K, *et al.* (2009) Receptor expression modulates the specificity of transforming growth factor-β signaling pathways. *Genes Cells* **14**, 469–482.

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