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

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Running head: Hepcidin expression in Mg deficient rats

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

Abbreviations: Bmp: bone morphogenic protein; Id1: inhibition of DNA binding 1; qRT-PCR, quantitative RT-PCR; TBARS, thiobarbituric acid-reactive substances.

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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 wk 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 morphogenic 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 Id1, a sensitive Bmp-responsive gene. In addition, the expression of Bmp receptors such as Alk2, Actr2a, Actr2b and 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 signaling 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.
Introduction
Mg is a co-factor 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, triglyceride 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 triglycerides 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 recognized as a hormone secreted from the liver in response to Fe overload; it negatively regulates intestinal Fe absorption through internalization 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 expression of hepatic Hepcidin in Mg-deficient rats.

Materials and methods
Animals and diets
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). They were individually housed in stainless steel cages in a temperature-, humidity- and light-controlled room (24°C, 60 %, 12 h light/dark cycle). All rats were fed a control diet (AIN-93G diet)\(^{15}\) for a 5-d adaptation period, followed by feeding either the control diet or an Mg-deficient diet (AIN-93G-based diet with Mg-free mineral mixture). The Mg content determined in the control diet and Mg-deficient diet was 49.6 mg/100 g and 4.2 mg/100 g, respectively. Rats were pair-fed their respective experimental diets and were allowed free access to demineralized water for 4 wk. After
the feeding trial, the rats were sacrificed by blood collection from the abdominal aorta under isoflurane anesthesia, and the liver was collected.

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

Diet, serum and liver samples were digested with trace-element grade nitric acid and hydrogen peroxide (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 homogenized in chilled saline by Polytron (PT1600E; Kinematica, Lucerne, Switzerland) and the homogenate was centrifuged at 105,000 \( \times \) g for 30 min at 4°C. Thiobarbituric acid-reactive substances (TBARS) concentration in the supernatant was determined by a commercial kit (OXI-TEK TBARS Assay Kit; ZeptoMetrix, 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 (qRT-PCR) was carried out as previously described\(^{16,17}\). The following oligonucleotides were used as PCR primers: 5’-gggcagaagaagcaagactgat-3’ and 5’-ttcagacttacatgcaagaggg-3’ for **Hepcidin** (Genbank accession number: NM_053469.1), 5’-gacagcagagtcgcaatcg-3’ and 5’-agctcaagtgagctcagtc-3’ for bone morphogenetic protein (**Bmp**)6 (Genbank accession number: NM_013107), 5’-gcgagatcagtgccttg-3’ and 5’-ttcagcttctgctctcag-3’ for inhibition of DNA binding 1 (**Id1**) (Genbank accession number: NM_012797.2), 5’-actctgtagggagtgc-3’ and 5’-actttcacaaagaggcacttg-3’ for **Hfe** (Genbank accession number: NM_053301.4), 5’-gtagcagagggagcag-3’ and 5’-tcaagggctgaggaagatt-3’ for **Hemojuvelin** (Genbank accession number: NM_001012080.1), 5’-gagttcagacactcaagca-3’ and 5’-tccagcctcaagagatt-3’ for transferrin receptor 1 (**Tfr1**) (Genbank accession number: NM_022712), and 5’-tcaaggtctgtgtc-3’ and 5’-gccggatacagcagctgtg-3’ for **Tfr2** (Genbank accession number: NM_001105916). PCR primers for activin receptor-like kinase (**Alk**)2, **Alk**3, activin receptor type IIA (**Actr**2a), activin receptor
type IIB (Actr2b), Bmp type II receptor (Bmpr2) and G3pdh were previously described\(^{(18)}\). The relative mRNA level was expressed as a ratio of the G3pdh mRNA level.

Statistical analyses
Data are expressed as the mean ± SEM. Differences between 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 TBARS, an index of oxidative stress, were higher in the Mg-deficient group. Expression of hepatic Tfr1 was significantly lower in Mg-deficient rats in control rats, whereas that of hepatic Tfr2 was comparable between 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 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
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 to a previous result; feeding a high Fe diet for 7 wk resulted in a 1.8-fold increase in Bmp6 expression and 7-fold 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 signaling\(^{(28)}\). Previous studies revealed that Hemojuvelin, which is a gene product of Hfe2 and a co-receptor of Bmps, 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 signaling, such as phosphorylation of Smad1/5/8 and Id1 expression, was not accelerated. Similar results were also recently obtained in patients with hereditary hemochromatosis 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 signaling 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 to in those from Hfe-null mice\(^{(32)}\).

Down-regulation of expression of Bmp receptors is possibly related to blunting of Bmp signaling 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 Alk6, a Bmp type I receptor, was not significant (data not shown). Receptor expression level also determines strength of the Bmp signaling\(^{(28,33)}\).

In conclusion, the accumulation of hepatic Fe by Mg deficiency is a stimulant inducing Bmp6 expression but not Hepcidin expression by blunting Bmp signaling 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.

10 Acknowledgements
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N.I. and T.M. designed the experiments; N.I. and M.K. performed the experiments; N.I., M.F. and T.M. analyzed data and wrote the paper. All authors discussed the results and approved the manuscript in its final version.

The authors declare no conflict of interest.

20 References


morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 38, 531-539.


Table 1 Effect of magnesium deficiency on serum concentration of magnesium, liver concentration of iron and thiobarbituric acid-reactive substances (TBARS), and hepatic expression of iron-related molecules

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mg deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Mg, mg/l</td>
<td>22.1 ± 1.7</td>
<td>7.3 ± 1.2**</td>
</tr>
<tr>
<td>Liver Fe, µg/g</td>
<td>87.8 ± 5.8</td>
<td>148.1 ±14.9**</td>
</tr>
<tr>
<td>Liver TBARS, nmol/g</td>
<td>35.9 ± 2.4</td>
<td>57.8 ± 1.7**</td>
</tr>
<tr>
<td><strong>Fe-related molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tfr1</td>
<td>1.00 ± 0.18</td>
<td>0.45 ± 0.11*</td>
</tr>
<tr>
<td>Tfr2</td>
<td>1.00 ± 0.04</td>
<td>0.93 ± 0.06</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM (n=6)
* and **P < 0.05 and 0.01, respectively, as compared to the control group.
Table 2 Effect of magnesium deficiency on hepatic expression of *Hepcidin*, bone morphogenetic protein (*Bmp*) 6, inhibition of DNA binding 1 (*Id1*), *Hfe*, *Hemojuvelin*, and Bmp receptors

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mg deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hepcidin</em></td>
<td>1.00 ± 0.13</td>
<td>0.98 ± 0.11</td>
</tr>
<tr>
<td><em>Bmp6</em></td>
<td>1.00 ± 0.29</td>
<td>2.22 ± 0.38*</td>
</tr>
<tr>
<td><em>Id1</em></td>
<td>1.00 ± 0.41</td>
<td>1.57 ± 0.72</td>
</tr>
<tr>
<td><em>Hfe</em></td>
<td>1.00 ± 0.05</td>
<td>0.70 ± 0.06**</td>
</tr>
<tr>
<td><em>Hemojuvelin</em></td>
<td>1.00 ± 0.21</td>
<td>1.66 ± 0.17**</td>
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</table>

Bmp receptors

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<table>
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<tbody>
<tr>
<td>Type I receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alk2</em></td>
<td>1.00 ± 0.15</td>
<td>0.44 ± 0.06**</td>
</tr>
<tr>
<td><em>Alk3</em></td>
<td>1.00 ± 0.10</td>
<td>0.70 ± 0.10</td>
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<tbody>
<tr>
<td>Type II receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actr2a</em></td>
<td>1.00 ± 0.09</td>
<td>0.55 ± 0.06**</td>
</tr>
<tr>
<td><em>Actr2b</em></td>
<td>1.00 ± 0.08</td>
<td>0.65 ± 0.09*</td>
</tr>
<tr>
<td><em>Bmpr2</em></td>
<td>1.00 ± 0.13</td>
<td>0.51 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM (n=6)

* and **: *P* < 0.05 and 0.01, respectively, as compared to the control group.