The impact of dietary magnesium restriction on magnesiotropic and calciotropic genes

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ABSTRACT

Background. Magnesium (Mg2+) is an essential electrolyte with important physiological functions. Consequently, hypomagnesaemia, an electrolyte disorder frequently diagnosed in critically ill patients, can have life-threatening consequences. The kidney plays a central role in the regulation of the Mg2+ balance. The present study investigated the molecular consequences of dietary Mg2+ restriction on renal Mg2+ transporters.

Methods. Two groups of 10 mice were fed a Mg2+-deficient diet or a Mg2+-enriched diet for 2 weeks. Serum and urine electrolyte concentrations were assayed. Next, renal mRNA expression levels of Mg2+-related genes were measured to determine their sensitivity to the dietary Mg2+ content. Subsequently, parvalbumin (PV) and the thiazide-sensitive Na+-Cl− cotransporter (NCC), both co-expressed in the distal convoluted tubule (DCT) with TRPM6, were further analysed at the protein level using immunoblotting and immunohistochemistry.

Results. Serum and urine electrolyte measurements revealed that dietary Mg2+ restriction resulted in significant reduction of serum Mg2+ and Ca2+ levels, and that the urinary excretion of these ions was also markedly reduced, while phosphate (P) excretion was significantly increased. In addition, the serum FGF23 level was markedly increased, whereas P was not significantly changed in the Mg2+-restricted mouse group. The renal abundance of hepatocyte nuclear factor 1 homeobox B (HNF1B) and the epithelial Mg2+ channel TRPM6 were increased in response to dietary Mg2+ restriction, whereas other magnesiotropic transporters were not affected. PV abundance was upregulated, while NCC was significantly downregulated. Furthermore, the expression levels of the epithelial Ca2+ channel TRPV5 and calbindin-D28K were markedly reduced in the low Mg2+ group.

Conclusions. Our data indicate an essential adaptive role for DCT during hypomagnesaemia since TRPM6, HNF1B, PV and NCC expression levels were adjusted. Moreover, hypomagnesaemia resulted in severe changes in Ca2+ and P reabsorption and expression levels of calciotropic proteins.

INTRODUCTION

Magnesium (Mg2+) is the most prevalent intracellular divalent cation. It plays a critical role in diverse biochemical and physiological processes, especially in neuromuscular functioning [1]. The plasma Mg2+ concentration represents 1% of total body Mg2+ and is regulated by the concerted action of intestinal absorption, storage in bone and reabsorption by the kidney [2]. Hypomagnesaemia is one of the most frequent electrolyte disturbances diagnosed in ~10% of hospitalized patients and can be as high as 65% in intensive care patients [3, 4]. Symptoms of hypomagnesaemia frequently involve neuromuscular irritability, such as tetany and seizures, and if not corrected in time, cardiac arrhythmias [5].
The kidney is the unfailing organ for Mg\(^{2+}\) homeostasis [6]. About 80% of the total plasma Mg\(^{2+}\) is ultrafiltrated through the glomerular membrane, of which 90–95% is reabsorbed in consecutive segments of the nephron [1]. Most of the Mg\(^{2+}\) reabsorption takes place in a passive paracellular manner in the proximal tubules (PTs) and the thick ascending limb of Henle (TAL) (10–30% and 40–70% of the filtered load, respectively). This transport is driven by a favourable concentration gradient for Mg\(^{2+}\) conditional on the Na\(^+-\)driven net water reabsorp-
tion in PT [7], and by the lumen-positive transepithelial potential in TAL, which is mainly generated by the Na\(^+\), K\(^+\), 2Cl\(^-\) cotransporter (NKCC2) [8]. Approximately 5–10% of the filtered load is reabsorbed by the distal convoluted tubule (DCT), where fine-tuning of Mg\(^{2+}\) reabsorption occurs via an active reabsorption process [2]. In this segment, the epithelial Mg\(^{2+}\) channel TRPM6 is conspicuously localized along the apical membrane, facilitating Mg\(^{2+}\) influx [9]. After the DCT, Mg\(^{2+}\) is not further reabsorbed and consequently around 1–3% of the filtered Mg\(^{2+}\) is excreted in the urine [1].

During the last decade, isolated cases of hereditary disorders of Mg\(^{2+}\) homeostasis have played a major role in the identification of magnesiotropic proteins localized in the kidney, like the discovery of TRPM6 in autosomal-recessive disorder hypomagnesaemia with secondary hypocalcaemia (HSH) [10, 11]. This resulted in the elucidation of additional genes responsible for the maintenance of Mg\(^{2+}\) balance, including the Na\(^+\), K\(^+\)-ATPase γ-subunit, a protein encoded by the FXYD2 gene [12]; the hepatocyte nuclear factor 1 homeobox B (HNF1B), which is shown to regulate the transcription of FXYD2 [13]; the KCNJ10 gene [14, 15], encoding the inwardly rectifying K\(^+\) channel 4.1 (Kir4.1) [16]; the epidermal growth factor (EGF) [17]; and finally, the KCNA1 gene, encoding the voltage-gated K\(^+\) channel 1.1 (Kv1.1) [18]. Moreover, in TAL mutations in genes encoding the interacting tight junction proteins, claudin-16 (CLDN16) [19] and claudin-19 (CLDN19) [20] were also linked to disturbances in Mg\(^{2+}\) homeostasis.

Previous studies have demonstrated that dietary Mg\(^{2+}\) restriction results in renal Mg\(^{2+}\) conservation, whereas a Mg\(^{2+}\)-enriched diet increases urinary Mg\(^{2+}\) excretion [21, 22]. The dietary Mg\(^{2+}\) content affected the renal TRPM6 expression on mRNA as well as protein level [22]. Alterations in the Mg\(^{2+}\) balance can also cause Ca\(^{2+}\) disturbances as a secondary adverse effect [23]. Indeed, Groenestege et al. [22] showed that dietary Mg\(^{2+}\) restriction not only leads to renal Mg\(^{2+}\) conservation, but also reduces Ca\(^{2+}\) wasting. Furthermore, the disease HSH displays a close interaction between Mg\(^{2+}\) and Ca\(^{2+}\) homeostasis. It has been suggested that hypocalcaemia is secondary to parathyroid hormone (PTH) failure resulting from Mg\(^{2+}\) deficiency [24]. However, the details of this mechanism remain largely unknown. Maintenance of the Ca\(^{2+}\) balance is, like for Mg\(^{2+}\), of vital importance for ample physiological functions [25]. The Ca\(^{2+}\)-sensing receptor plays a central role in the homeostasis of both divalent ions [26].

The aim of the present study was to investigate which magnesiotropic players are affected by the dietary Mg\(^{2+}\) content. Furthermore, the effect on other electrolyte transporters present in the TRPM6-expressing DCT segment was investigated. To this end, C57BL/6J mice were fed a Mg\(^{2+}\)-deficient (0.003% w/w Mg\(^{2+}\)) and -enriched (0.48% w/w Mg\(^{2+}\)) diet and serum and urine electrolyte measurements were performed for Mg\(^{2+}\), Ca\(^{2+}\), Na\(^+\) and K\(^+\). In addition, real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC) were used to determine the different mRNA and protein levels, respectively.

**MATERIALS AND METHODS**

**Animal studies**

C57BL/6J male mice (10 weeks of age) were purchased from Harlan/CPB (Zeist, The Netherlands) and maintained in a temperature-controlled and 12/12 h light-darkness room. To study the effect of dietary Mg\(^{2+}\), mice (n = 10 per group) were fed for 14 days a Mg\(^{2+}\)-deficient (0.0003% w/w) diet or a Mg\(^{2+}\)-enriched (0.48% w/w) diet (#TD.93106 and #TD.10531, respectively, Harlan-Teklad Madison, WI, USA). Both diets contained 0.6% w/w Ca\(^{2+}\), 0.45% w/w phosphorus and 2200 IU/kg vitamin D. The diets and deionized drinking water were provided *ad libitum*. A diet containing 0.19% w/w Mg\(^{2+}\) is considered normal for rodents [22]. Before the start of the experiment and on Day 12, the mice were individually housed in metabolic cages enabling 24-h urine collections (mineral oil was used to prevent evaporation) and to measure their water and food intake. At the end of the experiment, blood samples were taken under isoflurane (3.5% v/v) anaesthesia, and the mice were sacrificed by cervical dislocation. Subsequently, kidneys and colon were immediately frozen in liquid nitrogen for RNA and protein isolation or incubated in periodate-lysine-paraformaldehyde (PLP)-solution for IHC analysis. Blood was led to clot at room temperature, incubated overnight at 4°C and spun down for 5 min at 13 250 g, and the collected serum was used for analytical procedures. The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

**Analytical procedures**

Serum and urine Mg\(^{2+}\) concentrations were determined using a colorimetric assay according to the manufacturer’s protocol (Roche Diagnostics, Woerden, The Netherlands). Serum and urine Ca\(^{2+}\) concentrations were measured as described previously [27]. A flame spectrophotometer (FCM 6343; Eppendorf) was used to measure serum and urinary Na\(^+\) and K\(^+\) concentrations. Serum and urine phosphate (P\(^{3-}\)) concentrations were measured by the phosphomolybdate method with an Aerosef analyser (Abbott Diagnostics, Abbott Park, IL). Serum FGF23 levels were determined using an enzyme-linked immunosorbent assay kit (Immutopics, San Clemente, CA).

**Total kidney and colon RNA isolation and cDNA synthesis**

Total RNA was extracted from the kidney and colon using TriZol Total RNA isolation reagent according to standard procedures (Gibco BRL, Breda, The Netherlands). The obtained RNA was subjected to DNase treatment (Promega, Madison, WI) to prevent genomic DNA contamination. To determine
cDNA was used to determine the mRNA expression levels of genes of interest, as well as mRNA levels of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to design RT–qPCR primers according to the general criteria for these primers. All primer sequences used in this study are listed in Table 1. Prior to RT–qPCRs, the efficiency (95–105%) and dynamic range (R² > 0.98) were evaluated for each primer set. RT-PCRs were performed on a Biorad CFX96™ Real-Time PCR and Biorad C1000™ Thermal Cycler system. Reactions were performed in duplicate using a 6.25 µL SYBR®-Green Master Mix (Applied Biosystems, Foster City, CA), 12.5 ng of template cDNA and 400 nM of each primer in a final volume of 12.5 µL. All amplicons showed the correct sizes after gel electrophoresis and the dissociation curves showed one distinct melting peak, ensuring the absence of a nonspecific byproduct or primer dimers. Moreover, ‘No Reverse Transcription’ and ‘No Template’ controls were taken along.

### SYBR green real-time RT-qPCR

Real-Time PCR and Biorad C1000™ Thermal Cycler system. Reactions were performed in duplicate using a 6.25 µL SYBR®-Green Master Mix (Applied Biosystems, Foster City, CA), 12.5 ng of template cDNA and 400 nM of each primer in a final volume of 12.5 µL. All amplicons showed the correct sizes after gel electrophoresis and the dissociation curves showed one distinct melting peak, ensuring the absence of a nonspecific byproduct or primer dimers. Moreover, ‘No Reverse Transcription’ and ‘No Template’ controls were taken along.

### Table 1. Sequences of mouse primers used for an RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5′-TAACATCAAATGGGGTGAGG-3′</td>
<td>5′-GTGTTCACACCCCATCACAACAC-3′</td>
</tr>
<tr>
<td>TRPM6</td>
<td>5′-AAAGCCATGCGAGTTATCACG-3′</td>
<td>5′-CTTCACAATGAAAACCTGCCC-3′</td>
</tr>
<tr>
<td>TRPM7</td>
<td>5′-GGTGTTCCTCTGTGGTGTCCTT-3′</td>
<td>5′-CCCCATGTCGTCTTGTCGT-3′</td>
</tr>
<tr>
<td>Kv1.1</td>
<td>5′-CTGTGACAAATTGGAGGAAGATC-3′</td>
<td>5′-GAGCAACTGAGCCTGCTCTTC-3′</td>
</tr>
<tr>
<td>EGF</td>
<td>5′-GAGTGGCCCTGACTCTACCG-3′</td>
<td>5′-CCACATTGAGCAGGATAC-3′</td>
</tr>
<tr>
<td>EGFR</td>
<td>5′-CAGAACTGGGCTTAGGGAAC-3′</td>
<td>5′-GGACGATGTCCCTCCACTG-3′</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>5′-CCCGGATTATATCAGAGC-3′</td>
<td>5′-AGATCCCTGAGTGGAGCAA-3′</td>
</tr>
<tr>
<td>HNF1B</td>
<td>5′-CAAGATGTCAGGAGTGCCTAC-3′</td>
<td>5′-CTGGTCACTGAGCAGTGTTC-3′</td>
</tr>
<tr>
<td>FXYD2b</td>
<td>5′-GATCCCTGAGGTGCAAGGTCC-3′</td>
<td>5′-CCCTTGTGAGAATGATGAGG-3′</td>
</tr>
<tr>
<td>CLDN16</td>
<td>5′-GGTGGCCACACACATTAC-3′</td>
<td>5′-GAGGAGGGTCACGGTAAC-3′</td>
</tr>
<tr>
<td>CLDN19</td>
<td>5′-GGTGCCTCTCTGCTGAC-3′</td>
<td>5′-CCGGGCAAATCAAACAGC-3′</td>
</tr>
<tr>
<td>PV</td>
<td>5′-CGCTGAGGACATCAAGAAGG-3′</td>
<td>5′-CGGGGTTCTTTTTCTTACAGG-3′</td>
</tr>
<tr>
<td>NCC</td>
<td>5′-CTTCGGCCACTGCGGAC-3′</td>
<td>5′-GGTGGGTTCTTTCCCTTACAGG-3′</td>
</tr>
<tr>
<td>TRPV5</td>
<td>5′-CCACAGTGTAGCTGGGAGG-3′</td>
<td>5′-GAGATCTGTGTAAGATGAGG-3′</td>
</tr>
<tr>
<td>Calbindin-D28K</td>
<td>5′-CCACAGTGTAGCTGGGAGG-3′</td>
<td>5′-GAGATCTGTGTAAGATGAGG-3′</td>
</tr>
<tr>
<td>NHE3</td>
<td>5′-TGCTGGTGATCTGCTGAG-3′</td>
<td>5′-TCGCTGAGGTCCTCCACTC-3′</td>
</tr>
<tr>
<td>ENaC</td>
<td>5′-CATGCCTGGAGGATCAATG-3′</td>
<td>5′-CCATACAGGAGGTCCTCCATC-3′</td>
</tr>
</tbody>
</table>

Mouse primers used to perform SYBR Green RT-qPCR. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TRPM6, transient receptor potential melastatin member 6; TRPM7, transient receptor potential melastatin member 7; Kv1.1, voltage-gated K⁺ channel, shaker, member 1.1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; Kir4.1, inwardly rectifying K⁺ (Kir) channel 4.1; HNF1B, hepatocyte nuclear factor 1B; FXYD2b, FXYD domain-containing ion transport regulator 2, splice variant b; CLDN16, claudin-16; CLDN19, claudin-19; PV, parvalbumin; NCC, Na⁺,Cl⁻ cotransporter; TRPV5, transient receptor potential vanilloid member 5; Calbindin-D28K, Ca²⁺-binding protein D28K; NHE3, Na⁺-H⁺ exchanger 3: ENaC, epithelial Na⁺ channel.
were analysed with the Fiji ImageJ image analysis software (http://pacific.mpi-cbg.de).

**Immunoblotting**

Total kidney sections of mice treated with both diets were homogenized in buffer A (HbA; 20 mM Tris–HCl (pH = 7.4), 5 mM MgCl₂, 5 mM Na₂HPO₄, 1 mM EDTA, 80 mM sucrose, 1 mM PMSF, 1 μg/mL leupeptin and 10 μg/mL pepstatin). Protein concentration of the homogenates was determined by Bio-Rad Protein Assay, (Bio-Rad, Munich, Germany). The proteins were solubilized by 30' incubation at 37°C in Laemmli buffer. Sixty micrograms of each protein sample was separated on a 10% w/v sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel and blotted to a PVDF-nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA). Blots were incubated for 16 h with a rabbit HNF1B antibody (H-85, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or with a mouse tubulin antibody (1:20 000 dilution; Invitrogen, Camarillo, CA). Thereafter, blots were incubated with peroxidase-coupled secondary antibodies, and proteins were visualized using the chemiluminescence method (Pierce, Rockford, IL). Immunopositive bands were scanned using ChemiDoc XRS (Bio-Rad) and the signals were analysed using the Quantity One software (Bio-Rad). The amount of HNF1B protein was normalized for the corresponding total amount of protein, using tubulin protein levels.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical analyses were performed by an unpaired Student’s t-test. The low Mg²⁺ diet mouse group was compared with the high Mg²⁺ diet mouse group. P < 0.05 was considered statistically significant. All analyses were performed using the InStat 3 software.

**RESULTS**

**Serum and urine electrolyte levels of mice fed a low or high Mg²⁺ diet**

Before and after the diet period, mice were individually housed in metabolic cages to collect 24-h urine samples and to measure their water and food intake. Serum electrolyte concentrations, and urinary volumes and electrolyte levels are listed in Table 2. The mouse group fed with the Mg²⁺-deficient diet displayed a significant hypomagnesaemia accompanied by a marked hypocalcaemia compared with the mice receiving a Mg²⁺-enriched diet. Moreover, serum Na⁺ and K⁺ were slightly, but significantly affected by the Mg²⁺-deficient diet, Table 2. Serum electrolyte concentrations and urine analysis of mice following a Mg²⁺-deficient or Mg²⁺-enriched diet

<table>
<thead>
<tr>
<th>Measurement</th>
<th>0.003% w/w Mg²⁺</th>
<th>0.48% w/w Mg²⁺</th>
<th>Normal range²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Mg²⁺] (mmol/L)</td>
<td>0.63 +/- 0.04**</td>
<td>1.80 +/- 0.04</td>
<td>1.6–1.8</td>
</tr>
<tr>
<td>[Ca²⁺] (mmol/L)</td>
<td>1.94 +/- 0.02**</td>
<td>2.06 +/- 0.02</td>
<td>2.2–2.4</td>
</tr>
<tr>
<td>[Na⁺] (mmol/L)</td>
<td>139.6 +/- 0.8</td>
<td>136.2 +/- 0.5</td>
<td>130–150</td>
</tr>
<tr>
<td>[K⁺] (mmol/L)</td>
<td>7.60 +/- 0.07*</td>
<td>7.93 +/- 0.09</td>
<td>6–8</td>
</tr>
<tr>
<td>[P₀] (mmol/L)</td>
<td>2.4 +/- 0.2</td>
<td>2.0 +/- 0.1</td>
<td>2.2–2.7</td>
</tr>
<tr>
<td>[FGF23] (pg/mL)</td>
<td>157 +/- 19**</td>
<td>64 +/- 8</td>
<td>60–140</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mL/24 h)</td>
<td>1.3 +/- 0.1</td>
<td>1.2 +/- 0.1</td>
<td>~1.0</td>
</tr>
<tr>
<td>Mg²⁺ excretion (µmol/24 h)</td>
<td>0.22 +/- 0.04**</td>
<td>139 +/- 12</td>
<td>~30</td>
</tr>
<tr>
<td>Ca²⁺ excretion (µmol/24 h)</td>
<td>0.9 +/- 0.2**</td>
<td>23 +/- 2</td>
<td>~7.0</td>
</tr>
<tr>
<td>Na⁺ excretion (µmol/24 h)</td>
<td>91 +/- 6</td>
<td>76 +/- 9</td>
<td>~100</td>
</tr>
<tr>
<td>K⁺ excretion (µmol/24 h)</td>
<td>166 +/- 6</td>
<td>154 +/- 10</td>
<td>~200</td>
</tr>
<tr>
<td>P₀ excretion (µmol/24 h)</td>
<td>79 +/- 8*</td>
<td>1.9 +/- 0.3</td>
<td>~2.8</td>
</tr>
</tbody>
</table>

0.003% w/w Mg²⁺, mice receiving the deficient (0.003% w/w) Mg²⁺ diet; 0.48% w/w Mg²⁺, mice receiving the enriched (0.48% w/w) Mg²⁺ diet. Values are presented as means ± SEM.

²Normal serum values in C57BL/6J mice in mmol/L based on the values determined by Harlan (Harlan-Teklad Madison, WI), previous experiments performed in our lab and the metabolic cage data before the start of this experiment. The urinary excretion has a high variability.

³P < 0.05 compared with the high Mg²⁺ diet.

⁴P < 0.001 compared with the high Mg²⁺ diet.
resulting in an increased serum Na\(^+\) and a decreased K\(^+\) levels. In addition, the serum FGF23 level was markedly increased, whereas P\(_i\) was not significantly changed in the Mg\(^{2+}\)-restricted mouse group. Dietary Mg\(^{2+}\) restriction resulted in a substantial decreased urinary Mg\(^{2+}\) and Ca\(^{2+}\) excretion, whereas the Na\(^+\) and K\(^+\) excretions were not affected. Further, the urinary P\(_i\) excretion was markedly increased in the mouse group fed with the low Mg\(^{2+}\) diet (Table 2). Body weight, food intake, water intake and production of urine and faeces were not influenced by differences in the dietary Mg\(^{2+}\) content.

**Effect of dietary Mg\(^{2+}\) deficiency on mRNA expression of magnesiotropic transporters**

Table 3 shows the effect of dietary Mg\(^{2+}\) levels on renal mRNA abundance of TRPM6, the ubiquitously expressed Mg\(^{2+}\) channel TRPM7, Kv1.1, EGF, EGF receptor (EGFR), Kir4.1, HNF1B, FXYD domain-containing ion transport regulator 2, splice variants a and b (FXYD2a and FXYD2b), CLDN16 and CLDN19. The Mg\(^{2+}\)-deficient diet resulted in a significantly increased renal TRPM6 mRNA level. Interestingly, dietary Mg\(^{2+}\) restriction markedly upregulated the HNF1B level.

![Table 3](Image)

**Table 3. Effect of the dietary Mg\(^{2+}\) content on the expression profile of magnesiotropic genes in kidney**

<table>
<thead>
<tr>
<th>Gene</th>
<th>0.003% w/w Mg(^{2+})</th>
<th>0.48% w/w Mg(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPM6</td>
<td>204 ± 17(^%)</td>
<td>100 ± 7(^%)</td>
</tr>
<tr>
<td>TRPM7</td>
<td>95 ± 5(^%)</td>
<td>100 ± 7(^%)</td>
</tr>
<tr>
<td>Kv1.1</td>
<td>109 ± 8(^%)</td>
<td>100 ± 8(^%)</td>
</tr>
<tr>
<td>EGF</td>
<td>112 ± 5(^%)</td>
<td>100 ± 7(^%)</td>
</tr>
<tr>
<td>EGFR</td>
<td>86 ± 4(^%)</td>
<td>100 ± 6(^%)</td>
</tr>
<tr>
<td>Kir4.1</td>
<td>97 ± 7(^%)</td>
<td>100 ± 9(^%)</td>
</tr>
<tr>
<td>HNF1B</td>
<td>185 ± 19(^%)</td>
<td>100 ± 8(^%)</td>
</tr>
<tr>
<td>FXYD2b</td>
<td>103 ± 7(^%)</td>
<td>100 ± 4(^%)</td>
</tr>
<tr>
<td>CLDN16</td>
<td>99 ± 7(^%)</td>
<td>100 ± 5(^%)</td>
</tr>
<tr>
<td>CLDN19</td>
<td>100 ± 9(^%)</td>
<td>100 ± 10(^%)</td>
</tr>
</tbody>
</table>

A RT-qPCR was used to determine the mRNA expression levels of genes associated with Mg\(^{2+}\) homeostasis in total kidney lysate of mice on a Mg\(^{2+}\)-deficient diet (0.003% w/w), or an enriched Mg\(^{2+}\) diet (0.48% w/w). Expression levels are corrected for GAPDH and presented as a relative percentage of expression in mice put on the high Mg\(^{2+}\) diet. The mRNA levels of the following magnesiotropic genes were determined; the Mg\(^{2+}\) channels TRPM6 and TRPM7, the voltage-gated K\(^+\) channel Kv1.1, the EGF and its receptor (EGFR), the inwardly rectifying K\(^+\) channel Kir4.1, the hepatocyte nuclear factor 1B (HNF1B), the FXYD domain containing ion transport regulator 2, splice variant a (FXYD2a); the FXYD domain containing ion transport regulator 2, splice variant b (FXYD2b), claudin-16 (CLDN16) and claudin-19 (CLDN19). Values are presented as means ± SEM (n = 10).

\(^*\)P < 0.05 compared with the high Mg\(^{2+}\) diet.

However, differences in the dietary Mg\(^{2+}\) content did not significantly affect the renal abundance of TRPM6, Kv1.1, EGF, EGFR, Kir4.1, FXYD2a, FXYD2b, CLDN16 or CLDN19. Next, HNF1B protein levels of kidneys of the various dietary regimens were determined. In addition, low dietary Mg\(^{2+}\) significantly increased TRPM6 mRNA level in colon, compared with the high Mg\(^{2+}\) diet (Figure 1). Figure 2 depicts a representative immunoblot to detect HNF1B. The Mg\(^{2+}\)-deficient diet resulted in a markedly increased expression level of this protein (171 ± 19\(^\%\) and 100 ± 8\(^\%\) for low versus high Mg\(^{2+}\) diet, respectively, P < 0.05).

**Effect of dietary Mg\(^{2+}\) restriction on the expression levels of PV and NCC**

The mRNA expression level of PV was considerably increased (701 ± 34 and 100 ± 7\(^\%\) for low versus high Mg\(^{2+}\) diet, respectively, P < 0.05) (Figure 3A), whereas NCC was not significantly altered by the dietary Mg\(^{2+}\) regimen (105 ± 4 and 100 ± 5\% for low versus high Mg\(^{2+}\) diet, respectively, P > 0.05) (Figure 3B). Subsequently, immunohistochemical staining was performed to determine the expression of PV and NCC at the protein level. The Mg\(^{2+}\)-deficient diet resulted in a markedly increased expression level of PV (149 ± 4 and 100 ± 2\% for low versus high Mg\(^{2+}\) diet, respectively, P < 0.05) (Figure 3C), while the NCC protein levels were significantly downregulated (49 ± 5 and 100 ± 6\% for low versus high Mg\(^{2+}\) diet, respectively, P < 0.05) (Figure 3D), compared with the expression level in the mouse group fed the high Mg\(^{2+}\) diet.

**Effect of dietary Mg\(^{2+}\) deficiency on the expression levels of Ca\(^{2+}\) transporters, NHE3 and ENaC**

To investigate the underlying mechanism of the hypocalcaemia and sharp decline of urinary Ca\(^{2+}\) excretion of mice fed a low Mg\(^{2+}\) diet, the renal expression levels of TRPV5, calbindin-D\(_{28K}\), the Na\(^+-\)H\(^+\) exchanger 3 (NHE3) and the epithelial Na\(^+\) channel (ENaC) were determined. Figure 4 shows that dietary Mg\(^{2+}\) restriction results in significantly lower mRNA levels of TRPV5 and calbindin-D\(_{28K}\), compared with

![FIGURE 1](Image)

**Dietary magnesium restriction**

**FIGURE 1:** Effect of dietary Mg\(^{2+}\) content on the mRNA level of TRPM6 in colon. An RT-qPCR was used to determine the mRNA expression level of the epithelial Mg\(^{2+}\) channel TRPM6 in colon of mice on a Mg\(^{2+}\)-deficient diet (0.003% w/w, white bar), or an enriched Mg\(^{2+}\) diet (0.48% w/w, black bar). Expression level is corrected for GAPDH and presented as a relative percentage of expression in mice put on the high Mg\(^{2+}\) diet (0.48% w/w). Values are presented as means ± SEM (n = 10). \(P < 0.05\) compared with the high Mg\(^{2+}\) diet.
the kidneys of mice on the Mg$^{2+}$-enriched diet (45 ± 1 and 100 ± 7% for TRPV5 and 40 ± 2 and 100 ± 8% for calbindin-D$_{28K}$ for low versus high Mg$^{2+}$ diet, respectively, P < 0.05). In contrast, the mRNA levels of NHE3 and ENaC were not significantly affected by the dietary Mg$^{2+}$ content (93 ± 6 and 100 ± 7 and 99 ± 7 and 100 ± 4% for NHE3 and ENaC for low versus high Mg$^{2+}$ diet, respectively, P > 0.05). Next, the renal protein levels were determined for these calciotropic molecules. Semi-quantitative immunohistochemical analysis showed a strikingly low TRPV5 expression level (18 ± 2 and 100 ± 13% for low versus high Mg$^{2+}$ diet, respectively, P < 0.05) (Figure 4E) and calbindin-D$_{28K}$ (60 ± 3 and 100 ± 5% for low versus high Mg$^{2+}$ diet, respectively, P < 0.05) (Figure 4F) in the kidneys of mice fed a low Mg$^{2+}$ diet.

**DISCUSSION**

The present study demonstrated that dietary Mg$^{2+}$ restriction results in hypomagnesaemia accompanied by hypocalcaemia.
Moreover, the serum Na⁺:K⁺ ratio was slightly but significantly increased under dietary Mg²⁺ restriction. As renal adaptive response, the urinary excretion of Mg²⁺ as well as Ca²⁺ was markedly reduced in these mice. Interestingly, the urinary level of Pi was regulated in opposite direction compared with Ca²⁺ and also serum FGF23 was increased in the Mg²⁺-restricted mice. Analysis of expression levels revealed that the dietary Mg²⁺ content regulated TRPM6 and HNF1B abundance, while the other magnesiotropic transporters were not affected. In addition, the expression of TRPV5 and calbindin-D₂₈K was markedly lower in the Mg²⁺ restricted diet compared with the enriched Mg²⁺ diet. Finally, renal PV was upregulated and NCC downregulated as a consequence of the low Mg²⁺ diet. These results clearly indicate profound effects of Mg²⁺ restriction on gene expression levels in the distal part of the nephron.

The hypomagnesaemia and hypomagnesuria confirmed that the 2-week dietary Mg²⁺ restriction was effective. Furthermore, we revealed that the mRNA level of TRPM6 in kidney as well as in colon was upregulated during Mg²⁺ restriction. This suggests that a diminished Mg²⁺ excretion in kidney is accompanied by an enhanced absorption of Mg²⁺ in the large intestine. Whether the direct trigger for TRPM6 upregulation during a hypomagnesemic state is intrinsic (sensing of Mg²⁺ concentration by TRPM6 itself) or external (like hormonal regulation) remains elusive. Interestingly, the other magnesiotropic genes tested (TRPM7, Kv1.1, EGF, EGFR, Kir4.1, FXYD2a, FXYD2b, CLDN16 and CLDN19) were not affected by the amount of dietary Mg²⁺ intake. Although, we cannot exclude changes in the protein level of these players, these results emphasize the physiological importance of TRPM6, as a gatekeeper in Mg²⁺ balance. In our previous dietary studies, we did not observe a reduced serum Ca²⁺ level nor an increased level of TRPM6 in colon during Mg²⁺ restriction [22]. This difference can be due to the extended dietary period or to the more severe Mg²⁺ restriction in the current study. In addition, our data on TRPM6 regulation in kidney and colon are in line with the study of Rondon et al. [31].

HNF1B plays an essential role during renal embryonic development. Besides, it has been shown to be particularly important for the maintenance of Mg²⁺ and Ca²⁺ balance once matured, since mutations in HNF1B caused hypomagnesaemia and hypocalciuria [13]. The upregulation of HNF1B during dietary Mg²⁺ restriction is, therefore, of special interest. HNF1B regulates the transcription of FXYD2 [32], which

**FIGURE 4:** Effect of the dietary Mg²⁺ content on calciotropic mRNA and protein levels in kidney. An RT-qPCR was used to determine the mRNA expression levels of the epithelial Ca²⁺ channel TRPV5 (A) the cytosolic Ca²⁺-binding protein calbindin-D₂₈K (B) the Na⁺⁻H⁺ exchanger 3 (NHE3) (C) and the epithelial Na⁺ channel (ENaC) (D) in kidneys of mice fed a Mg²⁺-deficient diet (0.003% w/w), or an enriched Mg²⁺ diet (0.48% w/w). Expression levels are corrected for GAPDH and presented as a relative percentage of expression in mice on the high Mg²⁺ diet. TRPV5 and calbindin-D₂₈K protein abundance was determined by computerized analysis of immunohistochemical (IHC) images and presented as a relative percentage of the abundance in the high Mg²⁺ diet (E and F). The white bar indicates the Mg²⁺-deficient diet and the black bar the Mg²⁺-enriched diet. Values are presented as means ± SEM (n = 10 for RT-qPCR and n = 8 for IHC). * P < 0.05 compared with the high Mg²⁺ diet.
enodes the γ-subunit of the Na+, K+-ATPase. The latter trans-membrane protein has been shown to be important for the maintenance of Na, K+-ATPase capacity [33]. Probably, im-paired capacity to set the basolateral membrane potential, which ultimately hinders reabsorption of Mg2+ via TRPM6, seems to be the mechanism by which decreased expression of the γ-subunit resulted in hypomagnesaemia [32, 34]. However, the expression level of both FXYD2 transcription variants remained constant during the Mg2+- restriction. This suggests that HNF1B needs a co-factor to promote transcription of the γa-subunit or that HNF1B is responsible for main-taining the Mg2+ balance by other means.

The effect of dietary Mg2+ restriction is not limited to Mg2+- homeostasis since it seriously affected the renal handling of Ca2+ as well as of P, Pi is required for cellular functions and skeletal mineralization [35], and its homeostasis is closely related to that of Ca2+ [36].

The combination of a trend for increased serum P, significantly increased serum FGF23 and increased urinary P, excretion, suggests profound effects of dietary Mg2+ restriction on phosphate balance. Likely, the increased FGF23 level decreases P, reabsorption in the PT [37]. Borderline increased serum P, is the stimulus of the elevated FGF23 level in our mice [38]; however, the underlying mechanism for changes in serum P, remains elusive. The cascade observed in patients of persistent hypomagnesaemia causing hypoparathyroidism and consequently hypocalcaemia in combination with hyperphosphataemia [39, 40], is in line with our data.

Hypomagnesaemia in patients is frequently accompanied by hypocaliuria when the NCC activity is also diminished, i.e. Gitelman syndrome (GS) or chronic thiazide use [41]. Increased passive Ca2+ reabsorption in the PT, accompanied by an increased level of NHE3, responsible for Na+ and subsequent paracellular Ca2+ reabsorption [42], accounts for the underlying mechanism [43]. This is implicated via extracellular volume contraction and activation of the renin–angiotensin–aldosterone system (RAAS) [43]. In our study, the mRNA expression level of NHE3 was not affected by dietary Mg2+ intake, which implies that Ca2+ reabsorption in PT was not increased. Since, angiotensin II is a direct stimulator of ENaC activity [44], our observation that the ENaC mRNA level was not regulated further supports that RAAS signalling is not activated. However, this finding also implicates that the causative factor for the Na,K imbalances remains elusive.

The significant downregulation of TRPV5 and calbindin-D28K for which localization is restricted to DCT and connect-ing tubules (CNT), indicates that active Ca2+ reabsorption in these tubule segments is decreased. This could be the conse-quence of increased reabsorption of Ca2+ in TAL. Here, Ca2+ and Mg2+ transport compete for the same paracellular route. Given the hypomagnesaemia, a more favourable situation is possibly created to reabsorb Ca2+ in TAL [45]. Van Abel et al. [46] showed that the TRPV5 and calbindin-D28K expression levels are dependent on PTH stimulation. The reduced TRPV5 and calbindin-D28K levels as measured in the present study indicate a defect in the regulation of these proteins by PTH. Consequently, the co-existence of hypocalciuria and a decreased serum Ca2+ level in the low Mg2+ diet group suggests a disturbed bone metabolism. This is possibly induced by end-organ resistance to PTH also known as pseudohypoparathyroidism [47]. The latter, mediated by the continuously high levels of PTH, induced by the Mg2+-deficient state [48]. Disturbances in enzyme activity might form the underlying mechanism for the secondary effects on Ca2+ balance. Probably, defects in PTH secretion only occur after more prolonged periods of Mg2+- deficiency [49], like the experimental period of 2 weeks used in this study.

To obtain more insight into the function of DCT during hypomagnesaemia, the expression of other DCT-specific markers was investigated, including NCC and PV. PV is known as a Ca2+-binding protein with versatile functions in cellular processes [50] and was predominantly studied for its role in neurons and muscle fibres. In kidney, PV is exclusively expressed in the early DCT (DCT1), where it co-localizes with TRPM6 and NCC [9, 51]. To our knowledge, this is the first study showing a markedly increased mRNA and protein expression level of PV during dietary Mg2+ deficiency. Analysis of the renal phenotype of PV knockout mice showed polyuria and some aberrations in electrolyte homeostasis, however not for Mg2+ [51]. Interestingly, Belge et al. [51] observed reduced expression levels of NCC in the PV knockout mice, possibly mediated by diminished capacity to buffer intracellular Ca2+. NCC forms the apical entry mechanism for Na+ and Cl− in DCT. Mutations in the NCC gene cause GS, a salt-wasting renal disorder characterized by hypokalaemia, metabolic alkalosis, hypomagnesaemia and hypocalciuria [52]. Besides, NCC-deficient mice have a similar phenotype, including hypomagnesaemia [53]. This suggests that NCC is important for the maintenance of the Mg2+ balance. Interestingly, our data displayed that a Mg2+-deficient state results in decreased protein expression levels of NCC. Assembling these findings concerning PV and NCC, we hypothesize that hypomagnesaemia results in an increased [Ca2+]i, in DCT cells (resembling Ca2+ mobilization in heart or smooth muscle cells [49]) which downregulates the expression of NCC. Further, the upregulation of PV aimed to counteract the effect of [Ca2+]i, on NCC. The increased [Ca2+]i, might be mediated by a release from ER or mitochondria [54]. However, a role of [Mg2+]i, itself in the regulation of PV and/or NCC could not be excluded.

Figure 5 presents the main results of the present study and a schematic model of adaptations in the nephron in response to dietary Mg2+ restriction. In short, this study revealed that HNF1B and TRPM6 act as unique regulators of Mg2+ homeostasis. In addition, we observed more configurations during the hypomagnesemic state in DCT, namely changed PV and NCC expression levels. The detailed regulation of these proteins and their possible interaction via [Ca2+]i, remains to be established. Moreover, our results once again pinpoint the close interaction of the Mg2+ and Ca2+ balance. Downregulation of TRPV5 and calbindin-D28K combined with the decreased serum Ca2+ level, increased serum FGF23 level and increased urinary P, excretion in Mg2+- restricted mice suggests disturbances in control by PTH. Further experiments are needed to unravel the detailed triggers indu-cing the adaptations in DCT and the role of PTH during hypomagnesaemia.
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CONFLICTS OF INTEREST STATEMENT

None declared.

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