Transient Receptor Potential Melastatin 6 Knockout Mice Are Lethal whereas Heterozygous Deletion Results in Mild Hypomagnesemia

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Key Words
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Abstract
Background: Hypomagnesemia with secondary hypocalcemia is due to disturbed renal and intestinal magnesium (Mg²⁺) (re)absorption. The underlying defect is a mutation in the transient receptor potential melastatin type 6 (TRPM6), a Mg²⁺-permeable ion channel expressed in the kidney and intestine. Our aim was to characterize homozygous (–/–) and heterozygous (+/–) TRPM6 knockout mice with respect to Mg²⁺ homeostasis.

Methods: TRPM6 +/– mice were bred on a normal (0.19% wt/wt Mg²⁺) and high (0.48% wt/wt Mg²⁺) Mg²⁺ diet. In the offspring, 24-hour urinary Mg²⁺ and calcium excretion as well as serum concentrations of both were determined. TRPM6 mRNA expression in the kidney and colon was measured.

Results: On the regular diet, 30% of the offspring were TRPM6 wild-type (+/+), 70% were TRPM6 +/–, and none were TRPM6 –/–. The genotypic distribution of the litters remained the same on the 0.48% Mg²⁺ diet. In TRPM6 +/– mice on both diets, serum Mg²⁺ levels were significantly lower, and renal and intestinal TRPM6 mRNA expression was reduced. Urinary Mg²⁺ excretion was unaffected.

Conclusions: Homozygous TRPM6 deletion is embryonic lethal in mice. Heterozygous deletion of TRPM6 results in a mild hypomagnesemia. The Mg²⁺-enriched diet could not compensate for either embryonic lethality or hypomagnesemia caused by TRPM6 deficiency.

Introduction
Magnesium (Mg²⁺), the second most abundant electrolyte in the body, participates in various physiological processes like muscle function and enzyme reactions [1]. Mg²⁺ homeostasis is maintained by the kidney (excretion), intestine (absorption) and bone (storage) [2, 3]. In the intestine, Mg²⁺ uptake occurs via 2 distinct pathways, the active transcellular route and the passive paracellular way. The saturable transcellular route requires the entry of Mg²⁺ into epithelial cells by specific ion channels at the luminal site and extrusion at the basolateral membrane via yet unidentified exchangers. Under physiological conditions, paracellular absorption is the major route [3]. Following intestinal absorption, the kidney regulates the body’s Mg²⁺ balance. In the glomeruli, about 80% of Mg²⁺ is filtered, which is mainly reabsorbed passively in the proximal tubule and thick ascending limb of Henle’s...
loop. The minority of filtered Mg$^{2+}$ (5–10%) is reabsorbed in the distal convoluted tubule (DCT) in an active, tran-
scellular manner [4]. Mg$^{2+}$ reabsorption in the DCT de-
termines the final urinary excretion, because no reab-
sorption occurs beyond this nephron segment [2].

Various disorders are related to disturbances in Mg$^{2+}$ homeostasis. Hypomagnesemia with secondary hypo-
calcemia (HSH; OMIM 602014) is a rare, hereditary dis-
order caused by disturbed renal and intestinal Mg$^{2+}$ (re) absorption [5–8]. The disease clinically manifests during the first months after birth, and symptoms comprise generalized seizures. HSH is characterized by low-serum Mg$^{2+}$ concentrations, and hypomagnesemia results in secondary hypocalcemia in patients [9]. Therapy in-
cludes lifelong oral Mg$^{2+}$ administration to relieve clinical symptoms and to normalize calcium (Ca$^{2+}$) home-
ostasis, while serum Mg$^{2+}$ levels usually remain in the subnormal range (0.5–0.6 mmol/l, reference 0.7–1.1) [10]. Numerous patients suffer from diarrhea, a side effect of oral Mg$^{2+}$ supplementation [11]. Genetic screening of HSH patients identified mutations in transient receptor potential melastatin type 6 (TRPM6) as underlying de-
fect [8, 12]. TRPM6 belongs to the TRPM subfamily of ion channels. TRPM6 has been characterized as an Mg$^{2+}$-permeable ion channel [13], which contains an α-kinase domain at the carboxy (C) terminus [14, 15]. TRPM6 mu-
tations in HSH patients include point and missense mu-
tations, premature stops, inserted splice sites, frame shifts and exon deletions [8, 11, 12, 16–18]. The muta-
tions are spread over the entire TRPM6 gene, and most result in truncation of the TRPM6 protein. Recently, Chubanov et al. [16, 17] described 2 dominant negative TRPM6 missense mutations in HSH patients, S141L and P1017R, which directly affect the trafficking and func-
tion of this TRP channel.

In humans, TRPM6 is abundantly expressed in the co-
lon and kidney (DCT); both sites are associated with active Mg$^{2+}$ (re)absorption [12]. The expression pattern of TRPM6 in mice resembles that of humans, although TRPM6 is also highly present in the lung [19]. Colocaliza-
tion with the sodium-chloride cotransporter confirmed TRPM6 presence within the luminal membrane of DCT cells [13]. Dietary Mg$^{2+}$ restriction was shown to increase TRPM6 mRNA and protein expression in the mouse kid-
ney [19, 20], while Mg$^{2+}$ supplementation induces TRPM6 expression in the colon [19, 20]. TRPM6 expression and Mg$^{2+}$ (re)absorption are also regulated by a variety of hormo-
nes and compounds. Epidermal growth factor (EGF) and estrogen were discovered as magnesiotropic hormo-
nes directly affecting TRPM6 expression [19, 21]. EGF
enhances activation of TRPM6 via the EGF receptor and intracellular signaling cascades [22]. Recently, Glaude-
mans et al. [23] discovered a missense mutation (N255D) in the voltage-gated potassium channel Kv1.1 in a Brazilian family with isolated autosomal dominant hypomag-
nesemia. This Kv1.1 mutant depolarizes the membrane of the DCT, which reduces the driving force for Mg$^{2+}$ up-
take, resulting in renal Mg$^{2+}$ wasting in these patients [23].

Another TRPM family member, TRPM7, is the second Mg$^{2+}$ permeable ion channel known. TRPM7 is the closest homologue of TRPM6, but in contrast to TRPM6, the channel is ubiquitously expressed [15, 24]. Similar to TRPM6, TRPM7 contains an α-kinase domain at the C terminus [15, 24, 25]. In vitro and in vivo studies estab-
lished a role for TRPM7 in cell survival [25, 26]. Jin et al. [27] described that homozygous deletion of TRPM7 in mice results in embryonic lethality. Recently, Walder et al. [28] showed that TRPM6 knockout mice suffer from embryonic lethality and neural tube defects. Their study mainly focused on the role of TRPM6 in embryogenesis and less on the role of TRPM6 and/or TRPM7 in Mg$^{2+}$ homeostasis. Therefore, the aim of our study was to gen-
erate and characterize TRPM6$^{+/−}$ and TRPM6$^{−/−}$ mice in order to examine the role of TRPM6 and TRPM7 in renal and intestinal Mg$^{2+}$ handling in more detail.

Methods

Animal Studies

Male and female TRPM6$^{−/−}$ mice were purchased from The Jackson Laboratory (JAX Mice and Services, Bar Harbor, Me., USA; MGI: 2675603). These mice were bred to C57Bl/6J wild-type mice. The heterozygous offspring was bred to generate TRPM6$^{+/−}$ mice. TRPM6 littermates were housed in a temperature- and light-controlled room with standard pellet chow (0.19% wt/wt Mg$^{2+}$; SSNIFF Spezialitäten GmbH, Soest, Germany) or a Mg$^{2+}$-enriched diet (0.48% wt/wt Mg$^{2+}$; SSNIFF) and deionized drinking water available ad libitum. TRPM6 mice on the normal and high Mg$^{2+}$ diet (n = 10 per group) were housed in metabolic cages, and 24-hour urine and feces were collected. Blood samples were taken, and blood was led to clot at room temperature, incubated overnight at 4°C and spun down for 5 min at 13,250 g; the serum was used for analytical procedures. The animals were sacrificed, and kidney and colon tissues were sampled and frozen immedi-
ately in liquid nitrogen. Fresh urine was used for pH determina-
tion. Feces was dried for 8 h at 110 °C, incinerated for 5 h at 625 °C and resuspended in 2 M HCl overnight. The next day, the fecal solution was spun down for 15 min at 13,250 g, and the superna-
tant was used for the analytical procedures. All experiments were performed in compliance with the animal ethics board of the Radboud University Nijmegen.
Characterization of TRPM6 Knockout Mice

Genotyping

Genotypes were determined by PCR with primers A (5′-TGATTTCAGATGCATTCTCCAGG-3′) and B (5′-CATCCTCGTATAAGAAGGC-3′) to detect the wild-type allele, and C (5′-GCAGCGCATCGCCTTCTATC-3′) and B (5′-CATCCTCGTATAAGAAGGC-3′) to detect the null allele (fig. 1 a, adapted from JAX Mice and Services). The presence or absence of a 205-bp fragment (wild-type allele) or a 292-bp fragment (mutant allele) identifies animals with +/+ or –/− genotypes (fig. 1 b).

Quantitative Real-Time PCR Analysis

Total RNA was isolated from the kidney and colon using TriZol Total RNA Isolation Reagent (Gibco BRL, Breda, The Netherlands) as described previously [19]. cDNA was subsequently used to determine TRPM6 and TRPM7 mRNA expression levels. mRNA expression of the housekeeping gene hypoxanthine-guanine phosphorybosyl transferase was used as endogenous control. mRNA levels were quantified by qPCR on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Primers and fluorescent probes were purchased from Biolegio (Malden, The Netherlands), and the sequences are listed elsewhere [19].

Immunohistochemistry

Immunohistochemical staining of kidney sections with guinea pig anti-TRPM6 and semiquantitative determination of the protein levels were performed as described previously [19].

Analytical Procedures

Serum Mg²⁺ and urinary and fecal Mg²⁺ excretions were determined using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, The Netherlands). Serum, urine and feces Ca²⁺ were measured as described previously [29].

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical comparisons were analyzed by one-way ANOVA with a Bonferroni correction; p < 0.05 was considered statistically significant. All analyses were performed using the Statview Statistical Package software (Power PC version 4.51) on an Apple iMac computer.

Results

Homozygous Deletion of TRPM6 Is Embryonic Lethal

Breeding TRPM6+/− mice on a regular diet did not generate any TRPM6−/− offspring. Of 119 littermates that were born, 36 mice (30%) were genotyped as TRPM6 +/+, 83 (70%) were genotyped TRPM6 +/− mice, and 0 (0%) were genotyped as TRPM6 −/− (table 1). The average litter size was 6 (range 4–11). Since oral administration of Mg²⁺ significantly improves the clinical symptoms of HSH patients [10], a similar approach was used to generate TRPM6−/− mice. TRPM6+/− breeding pairs were fed a high Mg²⁺ diet, containing 2.5 times more Mg²⁺ than the regular diet. The content of all other elements was identical in both diets. The offspring was fed this diet after weaning until the end of the experiments. In total, 113 littermates were born (table 1), of which 29 (26%) were genotyped TRPM6+/+, 84 (74%) TRPM6+/− and 0 (0%) TRPM6−/− mice. We characterized the mice with a heterozygous deletion of TRPM6 and compared them with their wild-type littermates.
Mg$^{2+}$ and Ca$^{2+}$ Composition of Urine and Feces Is Not Altered in TRPM6$^{+/–}$ Mice

Mice between 8 and 10 weeks of age were housed in metabolic cages for 24 h, and urine and feces were collected to study Mg$^{2+}$ and Ca$^{2+}$ metabolism. The bodyweight of the TRPM6$^{+/–}$ mice on a regular or high Mg$^{2+}$ diet was similar to their wild-type littermates (table 2). Water intake, urine output and feces production in 24 h were comparable for all groups studied (table 2). Total urinary Mg$^{2+}$ and Ca$^{2+}$ excretion is shown in figure 2. TRPM6$^{+/–}$ and TRPM6$^{+/+}$ mice on control diet excreted the same amount of both electrolytes in 24 h (fig. 2a, b). Mice fed the high Mg$^{2+}$ diet excreted significantly more Mg$^{2+}$ and Ca$^{2+}$ in their urine compared with mice on the control diet (fig. 2a, b), but no significant differences between the 2 genotypes were observed. Urinary pH was not significantly affected by either heterozygous deletion of TRPM6 or by the high Mg$^{2+}$ diet (fig. 2c). Total fecal Mg$^{2+}$ and Ca$^{2+}$ excretion in 24 h was not significantly different between TRPM6$^{+/–}$ and TRPM6$^{+/+}$ mice (data not shown).

Heterozygous TRPM6 Deletion Causes a Mild Hypomagnesemia

On a regular diet, TRPM6$^{+/–}$ mice displayed a slight, but significantly lower serum Mg$^{2+}$ concentration compared with wild-type littermates (fig. 3a). Serum Mg$^{2+}$ levels in TRPM6$^{+/–}$ mice on a regular diet was 0.89 $\pm$ 0.01 versus 0.86 $\pm$ 0.01 mM for TRPM6$^{+/–}$ mice. Serum Ca$^{2+}$ concentrations were not altered in the TRPM6$^{+/–}$ mice.

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**Table 1.** Litter characteristics of TRPM6$^{+/–}$ breeding pairs on a regular or high Mg$^{2+}$ diet

<table>
<thead>
<tr>
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<th>Regular diet</th>
<th>High Mg$^{2+}$ diet</th>
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<tbody>
<tr>
<td>Total offspring</td>
<td>119 (100)</td>
<td>113 (100)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>36 (30)</td>
<td>29 (26)</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>83 (70)</td>
<td>84 (74)</td>
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<tr>
<td>Homozygote</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Litter size</td>
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<tr>
<td>Mean</td>
<td>6.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Range</td>
<td>4–11</td>
<td>2–9</td>
</tr>
</tbody>
</table>

Total offspring born from TRPM6$^{+/–}$ breeding pairs on a regular (0.19% wt/wt Mg$^{2+}$) or high Mg$^{2+}$ diet (0.48% wt/wt Mg$^{2+}$) arranged based on genotype. Data are expressed as absolute numbers and percentages (in parentheses) of total offspring born.

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**Fig. 2.** Effect of heterozygous deletion of TRPM6 on total urinary Mg$^{2+}$ and Ca$^{2+}$ excretion, and urinary pH. Net urinary excretion of Mg$^{2+}$ (a) and Ca$^{2+}$ (b) and urinary pH (c) in wild-type (+/+) or heterozygote (+/–) TRPM6 mice fed a regular diet (0.19% wt/wt Mg$^{2+}$) or a high Mg$^{2+}$ diet (0.48% wt/wt Mg$^{2+}$). Data are presented as the means $\pm$ SEM (n = 10 per group). *p < 0.05 versus TRPM6$^{+/+}$ on a 0.19% Mg$^{2+}$ diet; **p < 0.05 versus TRPM6$^{+/–}$ on a 0.19% Mg$^{2+}$ diet.
compared with wild-type littersmates (fig. 3b). TRPM6 /– mice fed a Mg 2+ -enriched diet also showed significantly lower serum Mg 2+ levels (0.83 ± 0.02 vs. 0.89 ± 0.01 mM for TRPM6 /+ mice; fig. 3a), whereas serum Ca 2+ concentrations were similar for both genotypes (fig. 3b). The serum Ca 2+ levels in TRPM6 /+ mice on the Mg 2+ -enriched diet are significantly reduced compared with those of mice on a control diet (fig. 3b).

**TRPM6 Gene Expression Is Reduced in TRPM6 /– Mice**

First, real-time PCR analysis was used to confirm the high TRPM6 mRNA expression in the kidney and colon of TRPM6 /+ mice (fig. 4a, d) [19]. In the kidney of TRPM6 /+ mice, TRPM6 mRNA levels were significantly lowered by 50 and 53%, respectively, compared with TRPM6 /+ mice on the control or Mg 2+ -enriched diet (fig. 4a). Renal TRPM6 protein expression was not significantly reduced in TRPM6 /+ mice on either diet (fig. 4b, c). TRPM6 gene expression in the colon was also significantly reduced by 54% in mice fed the high Mg 2+ diet (fig. 4d). On the regular diet, TRPM6 mRNA levels in the colon of TRPM6 /+ mice were lowered by 33% relative to TRPM6 /+ mice, an effect almost reaching significance (p = 0.0503; fig. 4d). TRPM7 gene expression in the kidney and colon was comparable for the 2 genotypes on the control and high Mg 2+ diet (fig. 4e, f). The Mg 2+ -enriched diet did not significantly change renal or intestinal TRPM6 or TRPM7 mRNA or protein levels (fig. 4).
Fig. 4. Effect of heterozygous deletion of TRPM6 on TRPM6 and TRPM7 gene and protein expression. TRPM6 (a, d) and TRPM7 (e, f) gene expression in the kidney (a, e) and colon (d, f), and renal TRPM6 protein expression [representative images (b); quantification of protein expression (c)] of wild-type (WT) (+/+) or heterozygote (+/−) TRPM6 mice on a regular diet (0.19% wt/wt Mg$^{2+}$) or a Mg$^{2+}$-enriched diet (0.48% wt/wt Mg$^{2+}$). Data are presented as the means ± SEM (n = 10 per group). $^a$ p < 0.05 versus TRPM$^{+/+}$ on 0.19% Mg$^{2+}$ diet; $^c$ p < 0.05 versus TRPM6$^{+/+}$ on 0.48% Mg$^{2+}$ diet. HPRT = Hypoxanthine-guanine phosphorybosyl transferase.
Discussion

Our study demonstrated that homozygous deletion of TRPM6 in mice leads to embryonic lethality, which could not be rescued by feeding the mice an a Mg^{2+}-enriched diet. Furthermore, the heterozygous deletion of TRPM6 resulted in a mild hypomagnesemia without hypocalcemia. The hypomagnesemia could not be prevented by the Mg^{2+}-enriched diet. In TRPM6^{+/–} mice, TRPM6 gene expression in the kidney and colon was downregulated, which most likely caused the reduction in serum Mg^{2+} levels. Our results stress the importance of TRPM6 in maintaining Mg^{2+} homeostasis.

Breeding TRPM6^{+/–} mice did not produce offspring that carried the homozygous deletion of TRPM6. Our observation suggests that TRPM6 plays an essential role in embryogenesis, which is in line with a recent publication by Walder et al. [28] and Jin et al. [27], describing embryonic lethality for the TRPM6^{−/−} and TRPM7^{−/−} mice, respectively. In humans, TRPM6 mutations result in hypomagnesemia, but no lethality is associated with TRPM6 dysfunction, even though most mutations result in truncation of the protein [8, 11, 12, 16–18]. This discrepancy could be related to different expression patterns in human and mouse, and TRPM6 expression in the human lung remains to be determined. Another possibility might be a consequence of the role of TRPM6 in embryogenesis in human or mouse. In mouse, TRPM6 is crucial during embryogenesis; its expression is peaking at day 10.5 after conception, and a complete lack of TRPM6 results in neu-
knockout mice can be used to study the consequences of TRPM6 deletion in the kidney, colon or both on the whole organism and might help elucidating the role of TRPM6 in the lung.

Hypomagnesemia most likely results from reduced TRPM6 mRNA expression in the kidney and colon in TRPM6+/– mice, although a significant reduction in renal TRPM6 protein expression could not be observed in our study, which leads to a significant decrease in serum Mg2+ levels, without a significant change in urinary Mg2+ excretion. No differences in renal or intestinal TRPM7 expression were observed in TRPM6+/– mice, suggesting that the decrease in Mg2+ (re)absorption is not compensated for by TRPM7. The Mg2+-enriched diet increased the urinary Mg2+ and Ca2+ content, which is in line with a previous publication by Groenestege et al. [19]. Serum Mg2+ concentrations and TRPM6 expression in the kidney and colon were not normalized by the high Mg2+ diet in TRPM6+/– mice. In TRPM6+/+ mice, TRPM6 gene and protein and TRPM7 gene expression in the kidney and colon were not affected by the Mg2+-enriched diet. Our results are partially in line with a previous publication, reporting no changes in renal TRPM6 and TRPM7 expression, but significantly enhanced TRPM6 gene expression in the colon of C57Bl6 mice by a Mg2+-enriched diet [19]. This discrepancy between the 2 studies might be explained by the timeframe of the experiment. In the previous report, the animals were fed the diet for a period of 10 days, starting at an age of 12 weeks [19], whereas in our study, mothers were fed the diet during breeding and weaning, and after weaning, the litters remained on the diet until the end of the experiments at an age of 8–10 weeks. This implies that mice respond quickly to an increased dietary Mg2+ intake, by enhancing TRPM6 mRNA expression in the colon, which stabilizes to control levels after a longer exposure to the diet.

In conclusion, our data indicate that homozygous TRPM6 deletion is embryonic lethal, as cross-breeding TRPM6+/– breeding pairs did not generate any TRPM6+/- mice. Feeding the breeding pairs a Mg2+-enriched diet could not compensate for the embryonic lethality caused by TRPM6 deficiency. Furthermore, our results clearly demonstrate the association of a mild phenotype with heterozygous deletion of TRPM6. TRPM6+/– mice show a significant reduction in TRPM6 expression in the kidney and intestine, which is accompanied by a mild hypomagnesemia. Overall, our results emphasize the key role of TRPM6 in Mg2+ homeostasis.

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References

Characterization of TRPM6 Knockout Mice


