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

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Abstract

Background: Hypomagnesemia with secondary hypocalcemia is due to disturbed renal and intestinal magnesium (Mg^2+) (re)absorption. The underlying defect is a mutation in the transient receptor potential melastatin type 6 (TRPM6), a Mg^2+-permeable ion channel expressed in the kidney and intestine. Our aim was to characterize homozygous (−/−) and heterozygous (+/−) TRPM6 knockout mice with respect to Mg^2+ homeostasis.

Methods: TRPM6 +/− mice were bred on a normal (0.19% wt/wt Mg^2+) and high (0.48% wt/wt Mg^2+) Mg^2+ diet. In the offspring, 24-hour urinary Mg^2+ 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% Mg^2+ diet. In TRPM6 +/− mice on both diets, serum Mg^2+ levels were significantly lower, and renal and intestinal TRPM6 mRNA expression was reduced. Urinary Mg^2+ excretion was unaffected.

Conclusions: Homozygous TRPM6 deletion is embryonic lethal in mice. Heterozygous deletion of TRPM6 results in a mild hypomagnesemia. The Mg^2+-enriched diet could not compensate for either embryonic lethality or hypomagnesemia caused by TRPM6 deficiency.

Introduction

Magnesium (Mg^2+), the second most abundant electrolyte in the body, participates in various physiological processes like muscle function and enzyme reactions [1]. Mg^2+ homeostasis is maintained by the kidney (excretion), intestine (absorption) and bone (storage) [2, 3]. In the intestine, Mg^2+ uptake occurs via 2 distinct pathways, the active transcellular route and the passive paracellular way. The saturable transcellular route requires the entry of Mg^2+ 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 Mg^2+ balance. In the glomeruli, about 80% of Mg^2+ is filtered, which is mainly reabsorbed passively in the proximal tubule and thick ascending limb of Henle’s loop.
loop. The minority of filtered Mg\(^{2+}\) (5–10%) is reabsorbed in the distal convoluted tubule (DCT) in an active, transcellular manner [4]. Mg\(^{2+}\) reabsorption in the DCT determines the final urinary excretion, because no reabsorption occurs beyond this nephron segment [2]. Various disorders are related to disturbances in Mg\(^{2+}\) homeostasis. Hypomagnesemia with secondary hypocalcemia (HSH; OMIM 602014) is a rare, hereditary disorder 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 includes lifelong oral Mg\(^{2+}\) administration to relieve clinical symptoms and to normalize calcium (Ca\(^{2+}\)) homeostasis, 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 defect [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 \(\alpha\)-kinase domain at the carboxy (C) terminus [14, 15]. TRPM6 mutations in HSH patients include point and missense mutations, premature stops, inserted splice sites, frame shifts and exon deletions [8, 11, 12, 16–18]. The mutations 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 function of this TRP channel.

In humans, TRPM6 is abundantly expressed in the colon 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]. Colocalization 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 kidney [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 hormones and compounds. Epidermal growth factor (EGF) and estrogen were discovered as magnesiotropic hormones directly affecting TRPM6 expression [19, 21]. EGF enhances activation of TRPM6 via the EGF receptor and intracellular signaling cascades [22]. Recently, Glaudemans et al. [23] discovered a missense mutation (N255D) in the voltage-gated potassium channel Kv1.1 in a Brazilian family with isolated autosomal dominant hypomagnesemia. This Kv1.1 mutant depolarizes the membrane of the DCT, which reduces the driving force for Mg\(^{2+}\) uptake, 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 \(\alpha\)-kinase domain at the C terminus [15, 24, 25]. In vitro and in vivo studies established 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 generate 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\(^{2+}\); SNIFF Spezialitaten GmbH, Soest, Germany) or a Mg\(^{2+}\)-enriched diet (0.48% wt/wt Mg\(^{2+}\); SNIFF) 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 immediately in liquid nitrogen. Fresh urine was used for pH determination. 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 supernatant 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'-TGATTGAGATGACACTGCA-3') and B (5'-CATTCTCTGTAAGAAAGGC-3') to detect the wild-type allele, and C (5'-GCAGCGCATCGCCTTCTATC-3') and B (5'-CATTCTCTGTAAGAAAGGC-3') to detect the null allele (fig. 1a, 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 +/+, –/– and +/– genotypes (fig. 1b).

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 Biologio (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 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+/– mice, 84 (74%) TRPM6+/– mice, and 0 (0%) TRPM6–/– mice. We characterized the mice with a heterozygous deletion of TRPM6 and compared them with their wild-type littermates.
Mg²⁺ and Ca²⁺ 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 Mg²⁺ and Ca²⁺ metabolism. The bodyweight of the TRPM6⁺⁻ mice on a regular or high Mg²⁺ 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 Mg²⁺ and Ca²⁺ 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 Mg²⁺ diet excreted significantly more Mg²⁺ and Ca²⁺ 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 Mg²⁺ diet (fig. 2c). Total fecal Mg²⁺ and Ca²⁺ 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 Mg²⁺ concentration compared with wild-type littermates (fig. 3a). Serum Mg²⁺ levels in TRPM6⁺⁻ mice on a regular diet was 0.89 ± 0.01 versus 0.86 ± 0.01 mM for TRPM6⁺⁻ mice. Serum Ca²⁺ concentrations were not altered in the TRPM6⁺⁻ mice.
compared with wild-type littermates (fig. 3b). TRPM6+/− mice fed a Mg2+-enriched diet also showed significantly lower serum Mg2+ levels (0.83 ± 0.02 vs. 0.89 ± 0.01 mM for TRPM6+/+ mice; fig. 3a), whereas serum Ca2+ concentrations were similar for both genotypes (fig. 3b). The serum Ca2+ levels in TRPM6+/+ mice on the Mg2+-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 Mg2+-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 Mg2+ 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 Mg2+ diet (fig. 4e, f). The Mg2+-enriched diet did not significantly change renal or intestinal TRPM6 or TRPM7 mRNA or protein levels (fig. 4).

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**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 phosphoribosyl 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 neural tube defects and lethality [28]. TRPM6 apparently plays a less significant role in humans in embryogenesis, since no significant link between miscarriages and TRPM6 families has been reported. Our approach to prevent the embryonic lethality is based on patient observations [10]. Increasing the Mg^{2+} intake enhanced the passive paracellular pathway of Mg^{2+} (re)absorption, which significantly improves hypomagnesemia in patients with HSH [10]. We reasoned that a similar approach might rescue the lethality of TRPM6^{−/−} mice. Therefore, TRPM6^{+/−} breeding pairs and their offspring were fed a Mg^{2+}-enriched diet. Increased urinary Mg^{2+} and Ca^{2+} excretion demonstrated the functionality of the diet (fig. 2a, b) without affecting the serum Mg^{2+} concentration (fig. 3a), confirming previous observations [19]. The litters consist of TRPM6^{+/+} and TRPM6^{+/−} mice only, hence no TRPM6^{−/−} mice survived embryogenesis, nor did we observe a high frequency of stillbirth or perinatal death. Thus, our theory was rejected, and the TRPM6^{−/−} mice could not be rescued with this approach. This is in contrast to the study by Walder et al. [28] in which 4 out of 105 offspring born from breedings on a high Mg^{2+} diet were TRPM6^{−/−}. The total number of offspring is comparable with our study (105 vs. 119); therefore, the birth of 4–5 TRPM6^{−/−} mice could have been expected based on the distribution reported by Walder et al. [28]. Since no TRPM6^{−/−} mice were obtained in our study, this suggests that TRPM6^{−/−} mice can survive sporadically. A difference in genetic background between the TRPM6^{+/−} mice used in the 2 studies might explain these variable observations, or it could be that the dietary Mg^{2+} supplementation (0.48% w/w) in our study was below a certain threshold and therefore could not rescue the TRPM6^{−/−} mice, since Walder et al. [28] acquired 4 TRPM6^{−/−} mice on a diet containing 0.6% w/w Mg^{2+}, which is slightly higher than in our experiments. Further investigations are required to understand this process. In our study, detailed analysis of embryonic lethality was not performed. Therefore, further research is required to elucidate this mechanism. It is interesting to speculate that the lethality is related to disturbances in Mg^{2+} homeostasis, since heterozygous deletion of TRPM6 leads to a mild hypomagnesemia in mice. Hypomagnesemia might become more severe and could be accompanied by hypocalcemia, similar to HSH patients, if both TRPM6 alleles are inactivated, although the results obtained with the TPRM7^{−/−} mice imply that other mechanisms than Mg^{2+} homeostasis are involved in premature death [27]. However, the expression pattern of TRPM6 suggests a more specific role for this ion channel in Mg^{2+} homeostasis than ubiquitously expressed TRPM7 [12, 15, 24]. The suggestion made by Walder et al. [28], linking Mg^{2+} to fetal malformations, might also explain the observed premature mortality. Another possible explanation for embryonic lethality might be related to the high expression of TRPM6 in the lungs of mice [19], although a function of TRPM6 in this tissue has not yet been described. In humans, TRPM6 expression in the lungs remains to be determined, nor has TRPM6 expression in the lung been linked to HSH so far. Generating conditional TRPM6^{+/−} mice in either a tissue- or developmental stage-specific manner would provide an animal model suitable for studying TRPM6 function in Mg^{2+} handling. Time- or tissue-specific TRPM6 knockout mice, either in single or several tissues, can be generated using the well-known Cre-LoxP recombinase system first described by Gu et al. [30]. Subsequently, cross-breeding of a kidney-specific TRPM6^{−/−} mouse with an intestine-specific TRPM6^{+/−} mouse will generate a mouse with targeted deletion of TRPM6 in both the kidney and colon. These conditional...
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 crossing 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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Characterization of TRPM6 Knockout Mice

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