Dietary Mg\textsuperscript{2+} regulates the epithelial Mg\textsuperscript{2+} channel TRPM6 in rat mammary tissue

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Abstract. The epithelial Mg\textsuperscript{2+} channel TRPM6 is considered a pivotal component in active Mg\textsuperscript{2+} absorption and re-absorption in the intestine and kidney, but its expression and function in other tissues are largely unknown. We have previously demonstrated that extracellular Mg\textsuperscript{2+} availability modulates TRPM6, but not the ubiquitous TRPM7, in cultured mammary epithelial cells; in addition, TRPM6 protein expression correlated to Mg\textsuperscript{2+} influx capacities. Our results closely remind the modulation of TRPM6 described by others in murine kidney and colon following Mg\textsuperscript{2+} dietary restriction. We sought to validate our observations by investigating whether TRPM6 modulation by extracellular Mg\textsuperscript{2+} also occurs in vivo. To this aim, we exploited a model consisting of rats fed either with a Mg\textsuperscript{2+}-deficient or a Mg\textsuperscript{2+}-enriched diet, and studied TRPM6 expression in breast and kidney tissues. Immunohistochemical and western blot analyses confirmed that rat mammary tissues express TRPM6 protein levels similar to those found in the kidney, and that protein expression is modulated by dietary Mg\textsuperscript{2+}. In particular, Mg\textsuperscript{2+} restriction upregulated TRPM6 expression, while Mg\textsuperscript{2+} supplementation resulted in a significant decrease in protein levels. This work confirms and extends our previous results on TRPM6 modulation by extracellular Mg\textsuperscript{2+} also occurs in vivo. To investigate this issue, a cellular model was established in our laboratory that consisted in mouse mammary epithelial cells adapted to non-physiological extracellular Mg\textsuperscript{2+} concentrations [3]. The characterisation of Low-Mg (growing at <25 \(\mu\)M Mg\textsuperscript{2+}) and High-Mg (growing at >40 mM Mg\textsuperscript{2+}) cultures showed that the expression of TRPM6 was significantly lower in the Low-Mg group compared to the High-Mg group. These findings suggest that TRPM6 plays a crucial role in the regulation of Mg\textsuperscript{2+} homeostasis in mammary tissue.

Key words: TRPM, magnesium deficiency, breast, magnesium transport

Recent studies have greatly increased our knowledge on the molecular regulation of intestinal and renal Mg\textsuperscript{2+} absorption/re-absorption [1, 2]. However, the mechanisms that regulate magnesium homeostasis in other tissues are not equally well known.

The absolute and universal requirement of magnesium for cell growth and metabolism implies that there must be precise control mechanisms that finely regulate Mg\textsuperscript{2+} concentration through a balance of Mg\textsuperscript{2+} uptake, intracellular storage and efflux. To investigate this issue, a cellular model was established in our laboratory that consisted in mouse mammary epithelial cells adapted to non-physiological extracellular Mg\textsuperscript{2+} concentrations [3]. The characterisation of Low-Mg (growing at <25 \(\mu\)M Mg\textsuperscript{2+}) and High-Mg (growing at >40 mM Mg\textsuperscript{2+}) cultures showed that the expression of TRPM6 was significantly lower in the Low-Mg group compared to the High-Mg group. These findings suggest that TRPM6 plays a crucial role in the regulation of Mg\textsuperscript{2+} homeostasis in mammary tissue.
Mg$^{2+}$) HC11 cells pointed out that, despite large in/out or out/in chemical gradients, these cells maintained their total magnesium content within a relatively narrow physiological range like their parental cells [3]. Nevertheless, in all three cell lines, total magnesium content increased during the exponential phase of cell proliferation, hence irrespective of extracellular Mg$^{2+}$ availability [4]. Altogether, these findings implied that HC11 cells must have developed specific mechanisms to maintain a total magnesium content compatible with survival, and to adjust it according to metabolic/proliferative requirements. In particular, this strongly suggested the existence of specialised Mg$^{2+}$ transport systems that can be modulated by magnesium availability and/or hormones, growth factors, etc. Indeed, we found that Low-Mg cells possess increased Mg$^{2+}$ influx capacity, while High-Mg cells display reduced influx and greater efflux capacity [5]. In the last decade there has been a burst of information on cation channels which could be rapidly modulated by changes in extracellular Mg$^{2+}$ availability, whereas TRPM7 expression remained unaltered [5].

TRPM6 was identified as a pivotal component in active Mg$^{2+}$ absorption and reabsorption in the intestine and kidney [7], which is consistent with its predominant expression in these tissues [8, 9]. Moreover, expression levels of TRPM6 resulted tightly regulated by dietary Mg$^{2+}$ in the same tissues. Dietary Mg$^{2+}$ restriction in mice significantly upregulated renal TRPM6 mRNA levels, whereas a Mg$^{2+}$ enriched diet increased TRPM6 mRNA expression in colon [9]. An analogous study [10] found that dietary Mg$^{2+}$ restriction increased TRPM6 expression both in kidney and colon, probably because of differences of experimental parameters such as diet composition and regimen duration. Interestingly, the two studies similarly found that TRPM7 mRNA expression was unperturbed in either tissue.

To our knowledge, our paper [5] was the first report of TRPM6 expression and modulation by extracellular Mg$^{2+}$ availability in mammary epithelial cells. Our results closely reminded the modulation of TRPM6 described in mouse kidney and colon following Mg$^{2+}$ dietary restriction [9, 10]. This prompted us to further investigate the matter, and clarify whether TRPM6 modulation could be considered an unsuspected as much as intriguing finding also in vivo. To this aim, we developed an in vivo model consisting of rats fed either with a Mg$^{2+}$-deficient or a Mg$^{2+}$-enriched diet, and studied TRPM6 expression in breast and kidney tissues by immunohistochemistry and western blot.

### Materials and methods

#### Animals and diets

The investigation conforms to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication no.85-23, revised 1996). Animal experiments were performed under licenses (experiment authorization number B-25-17 and protocol agreement B-25-056-6) obtained from the Veterinary Office of Franche-Comté (France). 12-week-old female Sprague-Dawley rats weighing 300 g (CHARLES RIVER, France) were placed in polyethylene cages and kept under constant temperature (22±2°C), constant humidity (50-60%) and a daily 12-hour light/dark cycle. Animals had free access to food and distilled water. After a 1-week acclimation period, rats were randomised into 3 groups (n=8 per group):

- “standard Mg” group (STD) was fed ad libitum for 6 weeks with a normal semisynthetic diet that comprised (per kg of diet): 200 g casein, 160 g cellulose, 400 g maize starch, 210 g granulated sugar, 25 ml corn oil, 25 ml peanut oil, 10 g vitamin mix and 140 g mineral mix. This diet corresponds to 1,000 mg/kg Mg;
- “Mg deficient” group (Mg-DEF) was fed ad libitum for 6 weeks with a Mg-deficient semisynthetic diet, that corresponds to 150 mg/kg Mg;
- “Mg supplemented” group (Mg-SUP) was fed ad libitum for 6 weeks with a Mg-enriched semisynthetic diet, that corresponds to 4,000 mg/kg Mg.

All diets were equivalent in terms of all compounds except magnesium mineral content. Magnesium was given in the form of Mg
monoxide/MgCl₂ (50/50). Diets were stored at room temperature, protected from light and distributed throughout the study.

**Body weight gain**

Rats were weighted at the beginning of the study (day 0) and every week throughout the study. The body weight gain was then calculated.

**Collection of blood and tissues**

At the end of the experimental protocol, rats were anesthetized with sodium pentobarbital (50 mg/kg bw, i.p.). Blood was collected via the abdominal aorta, immediately centrifuged at 5,000 g for 5 minutes at 4°C, and plasma was removed and stored at -20°C until analysis. Different tissues (mammary glands, ovaries and kidneys) were rapidly removed, weighed, immediately immersed in isopropanol for 5 minutes and finally frozen in liquid nitrogen. Tissues were stored at -80°C until analysis.

**Measurements of Mg²⁺ concentration**

The plasma was diluted with a solution of lanthanum oxide (0.1%) and analyzed by atomic absorption spectrophotometry (Perkin Elmer 3300) for determining plasma Mg²⁺ concentration.

**Immunohistochemistry**

Frozen tissue blocks were placed onto a base mold and covered with cryo-embedding medium (OCT). The base mold containing the tissue block was placed into liquid nitrogen and then transferred to a cryotome cryostat (-20°C). 5-μm-thick tissue sections were cut and mounted on clean glass slides. After air drying, sections were fixed in cold 3.7% paraformaldehyde and incubated in 0.3% H₂O₂ solution in methanol to block endogenous peroxidase activity. Specific binding sites were blocked first with 0.1% BSA in PBS and then with 10% goat serum. After blocking, slides were incubated overnight at 4°C with primary antibody anti-TRPM6 (rabbit polyclonal, Rb719-221108-WS Osenses, Australia) diluted 1:1,000 in goat serum blocking solution. Negative controls were incubated with PBS. After washing in PBS, all sections, including negative controls, were incubated for 40 minutes at 37°C with a biotinylated anti-rabbit secondary antibody diluted 1:200 in PBS. After washing, sections were immunostained using the avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) and peroxidase activity was visualized with the DAB substrate kit (Vector Laboratories). The slides were rinsed with H₂O and counterstained with Mayer’s hematoxylin. TRPM6 immunostaining was blindly evaluated by microscopic analysis. A percentage score system was used to differentiate TRPM6 expression. Slides showing no staining were considered negative. In positive slides, the overall intensity of staining was graded as follows: weak (33%), moderate (66%), and strong (100%). For each rat a mean score was calculated from two or more slides. Finally, the score was averaged for each condition (Mg-DEF, STD, Mg-SUP; n ≥ 3).

**Western Blot analysis**

Tissue samples were lysed in 50mM Tris pH 8, 150mM NaCl, 1 mM EDTA, 1% NP-40, 10 mg/mL leupeptin, 20 mg/mL aprotinin, 1mM PMSF, 1mM NaVO₄, 100mM NaF, and homogenized on ice with an Ultraturrax homogenizer. After incubation on ice for 90 minutes and centrifugation at 14,000 rpm for 15 minutes at 4°C, the supernatants were transferred to fresh tubes and protein concentration was determined by the Bradford assay (Bio-Rad). Protein samples (50 μg) were separated by SDS (8%)-polyacrylamide gel electrophoresis and blotted to polyvinylidene fluoride (PVDF) membrane. After transfer, membranes were blocked with a 5% solution of non-fat dry milk in Tris-buffered saline (pH 7.5) containing 0.5% Tween-20 (TBS-T) for 2 hours. Following blocking, membranes were incubated overnight at 4°C with the primary antibody: anti-TRPM6 diluted 1:2,000 (rabbit polyclonal, Rb719-221108-WS Osenses, Australia) or anti-ACTIN diluted 1:4,000 (rabbit polyclonal, Sigma). After washing, membranes were incubated for 90 minutes with horseradish peroxidase-conjugated secondary antibody (Amersham-GE Healthcare, Little Chalfont, Amersham) diluted 1:2,000. Bands were visualized by using the ECL Western Blotting detection system (GE Healthcare Life Science, Little Chalfont, GE Healthcare/Amersham). Densitometry was performed by ImageJ software (NIH, Bethesda, MD).
Statistical analysis

Data are presented as mean±SEM. All statistical analyses were performed by Prism 4 software (GraphPad Software Inc., San Diego, CA, USA), and differences were considered significant for p-values lower than 0.05.

Results and discussion

The present experiments were designed to study the effect of Mg2+ deficiency or supplementation on TRPM6 expression in mammary tissues in vivo. First of all, several parameters were evaluated to assess whether the different diets affected the status of the animals (table 1, figure 1). Rats on either Mg2+-restricted or -enriched diets showed essentially the same characteristics compared to rats fed with the standard diet (table 1). At present, we are not able to provide an explanation for the slightly significant discrepancy in the weight of the left kidney in Mg-SUP rats. The key parameter affected by diet was plasma Mg2+ concentration (figure 1). The magnesium-deficient diet significantly decreased Mg2+ concentration in plasma (64% vs control, p<0.001), while the magnesium-enriched diet resulted in a significant increase (180% vs Mg2+-deficient diet, p<0.001).

Preliminary western blot analyses suggested that rat mammary tissues express TRPM6 protein at levels comparable to those found in kidneys, and that renal levels of the channel are modulated by dietary Mg2+ intake (figure 2), as already shown in mice [9, 10]. In particular, low Mg2+ status determined by Mg2+ restriction seems to upregulate of renal TRPM6, which is indicative of an increase in transepithelial Mg2+ transport capacity to balance the low Mg2+ intake. On the contrary, Mg2+ supplementation did not appear to affect TRPM6 protein levels in the kidney.

TRPM6 protein expression was then analysed in mammary tissues from rats fed with the different diets. Mg2+-deficiency caused a slight upregulation of TRPM6 levels, as assessed either by immunohistochemistry (figure 3) or Western blot (figure 4), which is consistent with the overexpression we found in the in vitro HC11 model [5]. Interestingly, the Mg2+-enriched diet induced a significant decrease in mammary TRPM6 levels, which was also found in the in vitro HC11 model [5]. Other studies conducted in mice showed that Mg2+-deficiency induced a reduction

Table 1. Effects of dietary magnesium on body and tissue weights of rats on different Mg-containing diets.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dietary Mg (g/kg)</th>
<th>Body weight (g)</th>
<th>Body weight gain (g)</th>
<th>Right kidney weight (g)</th>
<th>Left kidney weight (g)</th>
<th>Right ovary weight (mg)</th>
<th>Left ovary weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg-DEF</td>
<td>0.15</td>
<td>329 ± 5</td>
<td>30 ± 5</td>
<td>0.99 ± 0.03</td>
<td>0.97 ± 0.04</td>
<td>98.5 ± 9.0</td>
<td>89.2 ± 7.3</td>
</tr>
<tr>
<td>STD</td>
<td>1</td>
<td>327 ± 3</td>
<td>28 ± 2</td>
<td>1.05 ± 0.03</td>
<td>0.99 ± 0.02</td>
<td>81.7 ± 6.5</td>
<td>81.5 ± 4.8</td>
</tr>
<tr>
<td>Mg-SUP</td>
<td>4</td>
<td>322 ± 11</td>
<td>30 ± 3</td>
<td>0.94 ± 0.04</td>
<td>0.86 ± 0.03*</td>
<td>89.3 ± 6.3</td>
<td>90.8 ± 3.1</td>
</tr>
</tbody>
</table>

Rats were weighted at the beginning of the study to assess net body weight gain. Data are mean±SEM (n=8 for STD and Mg-SUP groups, n=7 for Mg-DEF group). * p<0.05 vs STD by Tukey’s multiple comparison test following one-way analysis of variance (ANOVA).
Figure 2. Modulation of TRPM6 protein expression by dietary magnesium in rat renal tissues and comparison with TRPM6 levels in the breast, as assessed by western blot analysis. Tissues were from rats on standard (1 g/kg, STD), Mg-deficient diet (150 mg/kg, Mg-DEF) and Mg-supplemented (4 g/kg, Mg-SUP) diets. A representative blot and the relative densitometric quantification of TRPM6 protein normalized to actin are shown.

in TRPM6 expression, while the effects of Mg2+-supplementation were at variance [9, 10]. These discrepancies might be ascribed to species-specific characteristics or to differences in the experimental protocols. In any case, the cited studies only examined colon and renal TRPM6 expression, and we are not aware of any previous studies in breast tissues.

Overall, our results confirm that TRPM6 modulation by Mg2+ availability in mammary tissues occurs in vivo as well as in vitro. TRPM6 specifically regulates renal active Mg2+ re-absorption, consequently controlling plasma Mg2+ levels, thus it is not surprising that the low-Mg2+ status induced by dietary restriction results in upregulation of this channel in renal tissues. On the other hand, it is not straightforward to pinpoint the functional meaning of TRPM6 modulation in mammary tissues. Together with the epithelial origin, the breast and kidney might share the expression and modulation pattern of tissue-specific genes. It is intriguing, but at present a mere speculation, that TRPM6 may play a role in modulating Mg2+ absorption/re-absorption in mammary tissue following hormonal stimulation, e.g. during lactation. Two relevant crosstalk pathways have already been identified, that involve either the epidermal growth factor (EGF) [11, 12] or estrogen receptor (ER) [9, 13]. We plan future studies to correlate TRPM6 expression and mammary differentiation in the in vitro HC11 cellular model and in vivo, by analysing breast tissues.
Figure 3. Modulation of TRPM6 protein expression by dietary magnesium in rat mammary tissues as assessed by immunohistochemistry. A) Representative immunohistochemical staining of breast tissue sections from rats fed with standard (1 g/kg, STD), Mg-deficient (150 mg/kg, Mg-DEF), or Mg-supplemented (4 g/kg, Mg-SUP) diets. B) Quantification of immunohistochemical staining, as detailed in “Materials and methods”. Data are mean ± SEM (n≥3). ** p<0.01 vs Mg-DEF by two-tailed unpaired Student’s t test.

Figure 4. Modulation of TRPM6 protein expression by dietary magnesium in rat mammary tissues as assessed by western blot analysis. Tissues were from rats on standard (1 g/kg, STD), Mg-deficient diet (150 mg/kg, Mg-DEF) and Mg-supplemented (4 g/kg, Mg-SUP) diets. A representative blot and mean densitometric quantification of TRPM6 protein normalized to actin are shown. Data are mean±SEM (n≥4). ** p<0.01 vs Mg-DEF by two-tailed unpaired Student’s t test.
from lactating vs. non lactating animals held on different Mg\(^{2+}\)-containing diets. Moreover, breast tissues are known to express also the ubiquitous TRPM7 channel [14]. The coexistence and non-redundancy of both TRPM6 and TRPM7 in breast tissues call for deeper studies of their functional relationship. While it is still debated whether TRPM6 can form functional channel on itself [15, 16], it is well established that the two sister channels form heterotetramers with distinguishing biophysical characteristics from homotetramers [16-18]. The possibility exists that assorted TRPM homo- and heterotetramers may play different roles under various physiological and/or pathological conditions, and that their composition may accordingly vary. The alteration in the ratio between differently composed heteromers might constitute a flexible way to modulate cation influx and affect related signalling pathways in a tissue-specific manner.

**Conclusion**

This work confirms and extends our previous findings on TRPM6 modulation by Mg\(^{2+}\) availability in mammary epithelial cells to an in vivo setting. Further studies are required to clarify the functional significance of this channel in mammary tissues, and its role in tissue-specific magnesium homeostasis.

**Acknowledgments**

The authors wish to thank Marie-Chantal Canivenc (INRA Dijon, FRANCE) for her assistance in the collection of mammary glands.

**Disclosure**

Supported in part by the Italian MIUR-PRIN 2007ZT39FN. None of the authors has any conflict of interest to disclose.

**References**


