Dietary inulin in mice stimulates Mg\textsuperscript{2+} absorption and modulates TRPM6 and TRPM7 expression in large intestine and kidney

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Abstract. Complex fermentable carbohydrates, such as inulin-type fructans have been shown to improve Mg\textsuperscript{2+} absorption in the hindgut and body stores. The mechanisms for this are not well understood. The newly identified transient receptor potential melastatin 6 and 7 (TRPM6 and TRPM7) channels have been shown to function in active epithelial Mg\textsuperscript{2+} transport in the apical membrane of epithelial cells, the kidney and intestine and to be regulated by dietary intake. To determine the modulation of TRPM6 and TRPM7 expression in kidney and large intestine by long-chain inulin ingestion, C57B16J mice were fed a control or a long-chain inulin enriched diet (65 g of inulin/kg diet) for two weeks. Our results show that the inulin-enriched diet ameliorated Mg\textsuperscript{2+} absorption and Mg\textsuperscript{2+} bone stores. These features were accompanied by increased TRPM6 and TRPM7 expression in the hindgut. Downregulation of TRPM6 in the kidney of inulin fed mice could be related to reduced Mg\textsuperscript{2+} reabsorption and supports the beneficial effect of dietary fibers on Mg\textsuperscript{2+} absorption and stores. Inulin ingestion also modulates TRPM6 and TRPM7 expression in the large intestine. The origin and role of this modulation is not known. Changes in Mg\textsuperscript{2+} fluxes, lower pH of the digestive content and increased cell proliferation may be involved.

Key words: inulin, Mg\textsuperscript{2+} status, Mg\textsuperscript{2+} absorption, TRPM6, TRPM7

Numerous animal and human studies performed in our and other laboratories have pointed to the beneficial effects of complex fermentable carbohydrates on mineral absorption, particularly in improving Mg\textsuperscript{2+} absorption in distal parts of the intestine and hence ameliorating Mg\textsuperscript{2+} status [1-10]. Various mechanisms at the origin of this increased Mg\textsuperscript{2+} absorption in the large intestine have been proposed but not yet completely elucidated. In particular, in addition to the passive transport, the identity and role of specific transporters is not well known in the large intestine. Recently identified proteins - the transient receptor potential melastatin 6 and 7 (TRPM6 and TRPM7) channels have been shown to be essential for whole body and cellular Mg\textsuperscript{2+} homeostasis and to be regulated by dietary Mg\textsuperscript{2+} levels [11-15]. TRPM6 and TRPM7 potentially contribute to the maintenance of Mg\textsuperscript{2+} homeostasis since TRPM6 transports Mg\textsuperscript{2+} across the apical membrane of the epithelial cells in kidney and large intestine [11, 16] and the ubiquitously expressed TRPM7 regulates cellular Mg\textsuperscript{2+} uptake [12]. A strong link has been demonstrated between TRPM6 expression and Mg\textsuperscript{2+} status [15, 17].

Based on these data, we designed a study to determine the modulation of TRPM6 and TRPM7 expression in the kidney and large intestine by long-chain inulin ingestion in C57B16J mice. Inulin-type fructans were selected for this study because these fermentable carbohydrates have been shown
to efficiently increase Mg$^{2+}$ absorption from large intestine [9, 18]

**Materials and methods**

**Animals and diets**

Twenty four male C57B16J mice four months old were purchased from Janvier (Le Genest-Saint-Isle, France). During the first week of the experiment, the mice were fed a control diet (for composition see table 1). Then, the mice were randomly divided into two groups and over the following two weeks, each group received one of two diets: a control diet (the same as initially) or a diet enriched with long-chain inulin by substituting 10% of starch in the control diet with an equivalent quantity of long chain inulin from chicory (Inulin Beneo™ HP, generous gift from Beneo-Orafti, Tienen, Belgium) (table 1). The Mg$^{2+}$ and Ca$^{2+}$ content of the diets were 0.1% (wt/wt) and 0.4% (wt/wt) respectively. All diets were prepared in our laboratory. Distilled water and food were provided *ad libitum*. During the experiment, mice were housed one per cage (wire-bottomed to limit coprophagy) and maintained in a temperature-controlled room (22°C), with a 12-hour light/dark cycle. The last three days of the experiment the mice were housed in metabolic cages and food and water intake as well as urine and feces excretion were recorded daily. The animals were maintained and handled according to the recommendations of the Institutional Ethics Committee (INRA, Theix, France), in accordance with decree no. 87-848.

**Sampling procedures**

At the end of the experiment, the animals were sacrificed and blood (collected from the heart), bones (tibia), kidney, cecum and colon were harvested. The cecum, complete with content, was removed and weighed (total cecum weight). The caecal walls were cleaned with PBS, blotted on filter paper and weighed (caecal wall weight). All tissues (except bones) were cleaned from the blood and immediately frozen in liquid nitrogen and then stored at -80°C until analyses. Bones were cleaned from muscles and stored at 4°C.

**Plasma and Erythrocyte collection**

Blood from the heart was collected in heparin-containing tubes. Plasma was obtained by centrifugation (10 min, 3 500 rpm, 4°C). For erythrocyte Mg$^{2+}$ determination, erythrocytes were washed three times with saline solution and then hemolyzed in water-containing tubes. Both were frozen at -20°C for later analysis.

**Mineral Analysis**

Plasma, erythrocyte and urine Mg$^{2+}$ concentrations were determined after dilution with 0.1% (w/v) LaCl$_3$ as previously described [19, 20]. For tibia and feces the samples were weighed, dry-ashed (10 hrs at 500°C) and dissolved with concentrated HNO$_3$ (14M) and H$_2$O$_2$ (30%), on a heating plate until complete discoloration was achieved. The mineral solution was adjusted to 10 ml with distilled water and diluted with 0.1% LaCl$_3$. In the overall samples, Mg$^{2+}$ content was determined by atomic absorption spectrophotometry (using a Perkin-Elmer AA800, Quebec, Canada) at 285 nm.

**Creatinine analysis**

For creatinine measurement urine was diluted 20 times, and the creatinine concentration was determined using an automated chemical analysis kit, fol-

Table 1. Composition of experimental diets.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Control (g/kg)</th>
<th>Long-chain Inulin (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Corn starch</td>
<td>650</td>
<td>585</td>
</tr>
<tr>
<td>Inulin</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>Fiber alphacel</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mix*</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamins AIN 93A†</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>MgO</td>
<td>1.67</td>
<td>1.67</td>
</tr>
</tbody>
</table>

* Mineral mix composition (g/kg): calcium phosphate dibasic 500, sodium chloride 74, potassium citrate monohydrate 220, potassium sulphate 52, manganese carbonate 3.5, ferric citrate 6, zinc carbonate 0.53, cupric carbonate 0.1, sodium selenite 0.0033, chromium potassium sulphate 0.55, sucrose 135.3.
† AIN vitamin mixture (mg/kg): thiamine hydrochloride 600, riboflavin 600, pyridoxine hydrochloride 700, nicotinic acid 3000, D-calcium panthenothenate 1600, folic acid 200, D-biotin 20, cyanocobalamin (vitamin B$_{12}$) 1, retinyl palmitate (vitamin A) premix 1 600, DL-α-tocopherol-alumic acid 20 000, cholecalciferol (vitamin D$_3$) 250, menaquinone (vitamin K$_2$) 50, sucrose, finely powdered 972.9 g.
allowing the manufacturer’s instructions (Kone Progress Plus, Kone Instruments Oy, Espoo, Finland).

**Quantitative Real-Time PCR analysis**

Total RNA was extracted from complete segments of kidney, cecum and colon using the RNeasy Mini Kit (QIAGEN, Courtaboeuf, France), following the manufacturer’s protocol. Two μg of RNA was used for reverse transcriptase reactions with the Moloney murine leukemia virus Ready-To-Go You-Prime First-Strand Beads (Amersham, Orsay, France), following the manufacturer’s protocol. The mRNA levels of TRPM6 and TRPM7 were determined by SYBR Green quantitative real-time PCR in kidney, cecum and colon, using a Mastercycler ep realplex system (Eppendorf AG, Hamburg, Germany). The mRNA level of the hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as a control (considered as a housekeeping gene). Primers for the target genes used were obtained as previously described [15].

**Immunohistochemistry (IHC)**

Cryosections (7-μm thick) of periodate-lysine-paraformaldehyde-fixed kidney were stained as previously described [21-23]. Antiserum against TRPM6 [1:1,500 diluted with TNB buffer (TNT buffer containing blocking reagent)] (courtesy from R. J. Bindels, Nijmegen Centre for Molecular Life Sciences, The Netherlands) [22] (as primary antibody) and biotin-labeled, affinity-purified, goat anti-guinea pig IgG (1:2000; Sigma Chemical Co., St. Louis, MO) (as secondary antibody) were used. Micrographs of the cortex regions were acquired with an Olympus fluorescence microscope (Reichert-Jung Polyvar, Vienna, Austria) equipped with a Sony XC-71P CCD RGB digital camera (Kenmore, WA, US).

**Calculations and Statistical analysis**

Mg\(^{2+}\) absorption in this study was expressed as “net absorption”, which was calculated by substracting the Mg\(^{2+}\) intake (mg/d) from the Mg\(^{2+}\) excretion in feces (mg/d).

The apparent absorption rate as “absorption rate” was calculated by multiplying the Mg\(^{2+}\) net absorption (mg/d) to 100 and dividing it by Mg\(^{2+}\) intake (mg/d), the results are expressed as %.

Values are expressed as mean ± SE. Differences between the groups were tested by the Student’s t-test. Differences were considered significant at p < 0.05. The analyses were performed with the Sigmapr Stat 2.0 Statistical software (SPSS Inc, Chicago Illinois, USA).

**Results**

**Animal weight and cecum weight**

There were no significant differences in final body masses between the two groups studied (p = 0.14) (table 2). However, mice fed the long-chain inulin supplemented diet showed a significant increase in whole cecum weight (+ 143.9 ± 16.6%, p < 0.001), cecum content weight (+ 95.08 ± 26.3%, p = 0.003) and cecum wall weight (+ 265.7 ± 22.4%, p < 0.001) when compared to the control diet group (table 2).

**Intestinal absorption, urine excretion and balance of Mg\(^{2+}\)**

The parameters studied concerning Mg\(^{2+}\) balance evaluation are presented in table 3. In the present study mice were fed ad libitum and we did not observe any difference in total food intake of inulin supplementation (not shown), thus Mg\(^{2+}\) intake was similar in both groups (table 3). Fecal Mg\(^{2+}\) excretion was shown to be significantly lower in mice fed the inulin supplemented diet (- 27.0 ± 2.58%, p < 0.001) when compared to the control diet mice. Nevertheless, the calculated net Mg\(^{2+}\) absorption did not vary between the groups. A significant increase of Mg\(^{2+}\) absorption rate is evidenced in mice fed the inulin supplemented diet (+ 9.1 ± 1.1%, p < 0.001) when compared to the control group (table 3).

**Table 2.** Body weight and cecum weight in male mice fed a control or long-chain inulin supplemented diet for two weeks.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 12)</th>
<th>Long-chain Inulin (n = 12)</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Mouse weight (g)</td>
<td>27.09</td>
<td>0.37</td>
</tr>
<tr>
<td>Whole cecum weight (g)</td>
<td>0.24</td>
<td>0.02</td>
</tr>
<tr>
<td>Cecum content weight (g)</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>Wall cecum weight (g)</td>
<td>0.1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The results are expressed as means ± SE of 12 values per group. Student t-test was performed. *** p < 0.001, ** p < 0.01 vs control.

For details of diet and procedures, see “materials and methods”.
Twenty four hours urine Mg$^{2+}$ excretion was not significantly different in mice fed the inulin supplemented diet from that of controls. However, when correcting urine Mg$^{2+}$ excretion by creatinine, we evidenced an increased urine Mg$^{2+}$ excretion (+15.7% ± 6.2%) in mice fed the inulin supplemented diet as compared to controls (p = 0.04) (table 3). A slight but not significant increase of net Mg$^{2+}$ balance was observed in mice fed the inulin supplemented diet (table 3).

Blood and bone Mg$^{2+}$ content

Plasma and RBC Mg$^{2+}$ concentrations did not show any significant differences between the two groups studied (table 4). However, bone Mg$^{2+}$ content was significantly higher (+11.78 ± 1.65%, p < 0.007) in mice fed the inulin supplemented diet when compared to the control diet mice.

Table 3. Intake, faecal excretion, absorption, urinary excretion and net balance of Mg$^{2+}$ in male mice fed a control or long-chain inulin supplemented diet for 2 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 12)</th>
<th>Long-chain Inulin (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$ Intake (mg/d)</td>
<td>3.32 ± 0.14</td>
<td>3.19 ± 0.12</td>
</tr>
<tr>
<td>Mg$^{2+}$ Faecal excretion (mg/d)</td>
<td>0.84 ± 0.01</td>
<td>0.61*** ± 0.02</td>
</tr>
<tr>
<td>Mg$^{2+}$ Net absorption (mg/d)</td>
<td>2.43 ± 0.14</td>
<td>2.58 ± 0.12</td>
</tr>
<tr>
<td>Absorption rate (% intake)</td>
<td>73.95 ± 1.1</td>
<td>80.7*** ± 0.8</td>
</tr>
<tr>
<td>Mg$^{2+}$ Urinary excretion (mg/d)</td>
<td>0.78 ± 0.05</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Creatinine excretion (mg/d)</td>
<td>0.52 ± 0.03</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>Mg$^{2+}$ Urinary excretion/creatinine (μg/mg creatinine)</td>
<td>1523.7 ± 61.0</td>
<td>1762.4* ± 94.5</td>
</tr>
<tr>
<td>Mg$^{2+}$ Net balance (mg/d)</td>
<td>1.66 ± 0.14</td>
<td>1.74 ± 0.08</td>
</tr>
</tbody>
</table>

The results are expressed as means ± SE of 12 values per group. Student t-test was performed. *** p < 0.001, and * p < 0.05 vs control.

† For details of diet and procedures, see “materials and methods”.

Effect of dietary inulin on TRPM6 and TRPM7 expression in kidney and large intestine

Mice fed the inulin supplemented diet had a lower kidney TRPM6 expression than those fed a control diet (-21.2 ± 7%, p = 0.04) (figure 1A). IHC analysis of the kidneys confirmed that the inulin supplemented mice had lower levels of TRPM6 protein (figure 1B). Long-chain inulin supplementation did not significantly affect TRPM7 expression in the kidney (figure 1C).

Concerning the colon, no significant difference in TRPM6 and TRPM7 expression was observed in the ascendant colon when comparing the two groups (figure 2C, D). Nonetheless, inulin supplementation induced TRPM6 (+61.8 ± 11%, p = 0.003) and TRPM7 (+29.4 ± 8.6%, p = 0.01) expression in the descendant colon (figure 2E, F).

Discussion

Here we demonstrate that complex fermentable carbohydrate intake (in this case long-chain inulin) improves Mg$^{2+}$ absorption and consequently Mg$^{2+}$ stores in the body, as shown by a higher Mg$^{2+}$ content in bones. The consumption of this high-fiber diet and the subsequent Mg$^{2+}$ metabolism modifications are also accompanied by modulations in the expression of the two newly discovered Mg$^{2+}$ chan-
the kidney and large intestine.

In this study, several indices point to an improved Mg\(^{2+}\) absorption in animals fed an inulin-rich diet. This is in agreement with previously published work showing concomitant improvement of the Mg\(^{2+}\) absorption rate, decreased Mg\(^{2+}\) fecal excretion \([3, 5-8]\) and Mg\(^{2+}\) retention \([5, 10, 24]\) by inulin ingestion. As has been observed by others \([6]\), a slight but not significant increase was observed in Mg\(^{2+}\) net balances. In this study, the difficulty of observing a significant effect of inulin on Mg\(^{2+}\) balance is probably related to the short period of mineral balance and to the variability of measured parameters. However, as we and others observe \([7, 25]\) the increased Mg\(^{2+}\) absorption is translated into increased Mg\(^{2+}\) bone stores. In concordance with previously published studies \([2, 6, 24, 26, 27]\), no changes were observed in plasma and erythrocyte Mg\(^{2+}\) levels. This undoubtedly results from a homeostatic adaptation because animals in the present study were fed a Mg\(^{2+}\) adequate diet.

The increased Mg\(^{2+}\) absorption results from the effect of the high-fiber diet on the large intestine. The inulin-rich diet in the present study, and as has been described before led to a cecal weight rise, due to the increase in cecal content and cecal wall weight \([24, 27, 28]\). In fact, the inulin-type fructans are non digestible oligosaccharides \([29-31]\). Because of their \(\beta-(2\rightarrow1)\) fructosyl-fructose linkages, inulin-type fructans resist enzymatic hydrolysis in small intestine and are fermented in the large intestine. The mechanisms involved in the increased Mg\(^{2+}\) absorption in the intestine are still a matter of discussion. Acidification of the luminal contents and greater solubilization by SCFA production \([32]\), hypertrophy of the intestinal mucosa \([6, 18]\), and increased paracellular and transcellular transport may lead to an increase in Mg\(^{2+}\) uptake by the intestinal cells \([32-34]\).
Figure 2. TRPM6 and TRPM7 expression in the large intestine of mice fed a control or inulin supplemented diet. TRPM6 and TRPM7 mRNA levels measured by RT-PCR, respectively in cecum (A, B); in ascendant colon (C, D) and in descendant colon (E, F). For the RT-PCR, the mean value of the control group was considered 100% and the results are expressed as percentage means (± SE) of 9-12 values per group. Student t-test was performed. ** p < 0.01 and * p < 0.05 vs control.
Interestingly, in this study, it is evidenced for the first time that stimulated Mg\(^{2+}\) absorption, and consequently Mg\(^{2+}\) status, by supplementation with complex fermentable carbohydrates is accompanied by upregulation of TRPM6 and TRPM7 expression in the distal parts of the intestine. Similarly, Groenestege et al. [15] observed that Mg\(^{2+}\) supplementation induced TRPM6 expression in the colon.

These observations point to the possible regulation of TRPM6 expression by complex fermentable carbohydrates and thus an induced high Mg\(^{2+}\) status, however, it is not clear if this effect is direct or indirect. It is known that the saturable absorptive process functions only at very low dietary Mg\(^{2+}\) intakes, however, it is not well known in the large intestine [35]. Another mechanism for this could be acidification of the lumen induced by SCFA production. In fact it has been shown that these two channels are pH sensitive [36] but the effect of on their expression is not known.

In this study we also observed that TRPM7 expression was induced in the cecum and colon of inulin-fed mice. In addition to mechanisms discussed above, this may have a link with the strong proliferative effect of dietary fiber [2, 37]. Considering that TRPM7 is a Mg\(^{2+}\)-permeant channel responsible for transcellular Mg\(^{2+}\) transport and an important mediator of cell growth [38, 39] it can be hypothesized that TRPM7 upregulation could be a response for increased cell proliferation and growth.

In the present work we have also shown that neither TRPM6 nor TRPM7 kidney expression was lower in inulin-fed mice, as compared to controls. The downregulation of TRPM6 expression is an expected homeostatic response to the increased Mg\(^{2+}\) absorption. This is in agreement with results from studies on Mg\(^{2+}\) supplemented animals [15, 17]. It is also well known that complex fermentable carbohydrates may also stimulate Ca\(^{2+}\) absorption [32]. The impact of this effect on different gene expression, such as TRPM6 and TRPM7, is not defined in this particular condition.

In conclusion, inulin ingestion leads to an improved Mg\(^{2+}\) absorption and modulates TRPM6 and TRPM7 expression in the distal parts of the large intestine. The downregulation of TRPM6 in the kidney supports the beneficial effect of dietary fibers on Mg\(^{2+}\) absorption and stores. The origins and roles of the modulation of TRPM6 and TRPM7 expression in the large intestine are not known. Changes in Mg\(^{2+}\) fluxes, lower pH of the digestive content and increased cell proliferation could be involved.

Acknowledgments

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References


INULIN MODULATES TRPM6 AND TRPM7 EXPRESSION


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