Decreased renal mRNA expression of TRPM6 is associated with hypomagnesemia in C57BL/6 asthmatic mice

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Abstract. – Background and Objectives: Hypomagnesemia has been reported up to 40% in asthma patients, and a relationship between hypomagnesemia and asthma severity has been previously characterized. However, the mechanism for hypomagnesemia in asthma patients is not clear. Transient receptor potential melastatin 6 (TRPM6) is a newly identified channel that is involved in active epithelial magnesium transport, and downregulation of TRPM6 in the kidney was related to reduced Mg2+ reabsorption in mouse model. The aim of the study was to investigate whether plasma and erythrocyte magnesium levels were correlated with renal expression of TRPM6 mRNA in C57BL/6 asthmatic mice.

Materials and Methods: 48 healthy C57BL/6 mice were randomly divided into asthmatic group and control group with 24 mice in each group. Each group were randomly taken out 8 mice at 1d, 21d, 34d to detect plasma Mg2+, intracellular Mg2+ and renal TRPM6 mRNA expressions.

Results: There were no significant difference in plasma Mg2+, intracellular Mg2+ and TRPM6 mRNA expression of renal tissues between asthmatic group and control group at 1d. However, plasma Mg2+ and intracellular Mg2+ as well as TRPM6 mRNA of renal tissues in asthmatic group were significantly lower than that of control group at 21d and at 34d. Both plasma Mg2+ and intracellular Mg2+ were positively correlated with TRPM6 mRNA expression in the renal tissues.

Conclusion: This study indicates that the consistently reduced expression of TRPM6 mRNA may play a role in the pathogenesis of hypomagnesemia in C57BL/6 asthmatic mice.

Key Words: C57BL/6 mice, Asthma, Magnesium, Transient receptor potential melastatin 6, Kidney.

Introduction

Magnesium (Mg) is the second most abundant intracellular cation and plays a crucial role in many biochemical and physiological processes. It is involved in the modulation of smooth muscle contraction and hypomagnesemia results in an increase in bronchi smooth muscles excitability and their contraction. Asthma is a chronic respiratory disease characterized by increased bronchial smooth muscle contractility and consequent bronchial hyperactivity. There are numerous researches on the relationship between hypomagnesemia and asthma patients, yet the results remain controversial. However, a recent report shows a significantly decreased magnesium to calcium ratio in asthma children as compared with normal controls, suggesting a pathologic magnesium state in asthma.

Magnesium homeostasis mainly depends on the balance between intestinal absorption and renal excretion. Although the mechanisms for regulation of magnesium absorption remain unknown, recent studies have shown an important role of transient receptor potential melastatin 6 (TRPM6) in this process. TRPM6 is a newly identified member of the long transient receptor potential channel (TRPM) family and proved to be involved in active epithelial magnesium transport in intestine and kidney. Downregulation of TRPM6 in the kidney has been proved to be related to reduced Mg2+ reabsorption in inulin fed mice and suppressed TRPM6 expression by siRNA results in a decrease of renal Mg2+ reabsorption mediated by inhibition of c-Fos transcription.
At present, little is known about whether TRPM6 is involved in the decreased magnesium levels in some asthmatic patients. The aim of the study was to evaluate whether there was hypomagnesemia in C57BL/6 asthmatic mice, and explore the possible role of TRPM6 if there was a magnesium deficiency.

**Materials and Methods**

**Experimental Animals**

C57BL/6 mice (all females, 4-6 weeks old) were purchased from the Animal Centre of Zhongshan University, P.R. China. Mice were given standard laboratory rodent chow and water. All experimental procedures were approved by the local Ethics Committee for animal experiments (Faculty of Medicine, Zhongshan University). The mice were randomly divided into asthmatic group (n = 24) and control group (n = 24).

**Allergen Exposure Protocols**

Mice were sensitized by an intraperitoneal injection of 0.2 ml of alum-precipitated antigen containing 50 µg of ovalbumin (OVA grade VI, Sigma-Aldrich) absorbed to 5 mg of alum (aluminium hydroxide hydrate gel, LSL) in saline vehicle on day 1 and 14, and then challenged with 0.2% OVA through the nasal route in a volume of 50 µl every day from day 15 to day 21 and every two days from day 22-34. The control mice were sensitized and challenged with normal saline instead.

**Collection of Samples**

12 hours after the final allergen challenge, mice were anesthetized by intraperitoneal injection of 0.2 ml of alum-precipitated antigen containing 50 µg of ovalbumin (OVA grade VI, Sigma-Aldrich) absorbed to 5 mg of alum (aluminium hydroxide hydrate gel, LSL) in saline vehicle on day 1 and 14, and then challenged with 0.2% OVA through the nasal route in a volume of 50 µl every day from day 15 to day 21 and every two days from day 22-34. The control mice were sensitized and challenged with normal saline instead.

**Examination of Plasma and Erythrocyte Magnesium Levels**

Blood samples were left for 30 min at room temperature, and plasma was obtained after centrifugation at 2500 rpm for 15 minutes. These samples were stored at -80°C until use. Erythrocytes were washed by equal volume of saline and centrifuged at 2500 rpm for 5 minutes. This procedure was repeated twice and the last centrifugation lasted for 40 minutes. Add 50 µl erythrocytes to 150 µl distilled water and mix by vortexing, and kept the upper layer for further assays. Plasma and erythrocyte magnesium concentrations were analyzed by means of atomic absorption spectrophotometry.

**RNA Isolation and RT-PCR**

Total RNA was isolated from renal tissues using TRIzol reagent (Invitrogen) and reverse transcription was performed by First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Harrington, Ont., Canada) using 2 µg of total RNA according to the manufacturer’s instructions. The resulting first-strand cDNA of reverse transcription reaction was directly used for PCR amplification.

The protocol for TRPM6 PCR amplification consists of denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. This was followed by a final extension at 72°C for 10 min. Products were separated by 2% agarose gel electrophoresis and visualized by colloidal gold staining. Scion image software was applied and the relative density of TRPM6 compared to that of actin were analyzed. Primer sequences were as follows: TRPM6: 5'-CCACCAATACCCCTGGAAGAA-3' (forward), 5'-AGGAGTTGCAGCGATGTTT-3' (reverse); actin: 5'-GTTGGTTGGAAACATCC-3' (forward), 5'-AAGCAATGCTGCTACCTTCC-3' (reverse).

**Statistical Analysis**

All data are demonstrated as means±standard error of mean (SEM). Differences between groups of data were explored using one-way ANOVA or Student’s paired or unpaired t test (two-tailed) as appropriate. Correlation between two measurement variables was evaluated by linear correlation analysis. Data were analysed with the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Comparison of Plasma Magnesium Levels in Asthmatic Group and Control Group**

Table I shows the plasma magnesium profile in the asthmatic group and the control group. At day 1, no significant difference was found be-
between the two groups ($t=0.642$, $p=0.531$). At day 21 and day 34, the mean plasma magnesium levels were significantly lower in asthmatic group than in control group ($t=5.85, 3.29$), respectively, $p<0.05$. For the control group, there was no significant difference in plasma magnesium concentrations at day 1, day 21 and day 34 ($F=1.096, p=0.353$). For the asthmatic group, significant difference in plasma magnesium concentrations were found among day 1, day 21 and day 34 ($F=11.657, p=0.000$): the plasma magnesium concentrations at day 34 were significantly lower than that of day 1 and day 21 ($q=4.35, 3.98$, respectively, $p<0.05$).

Table I. Comparison of plasma Mg$^{2+}$ between asthmatic group and control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>1d (mmol/L)</th>
<th>21d (mmol/L)</th>
<th>34d (mmol/L)</th>
<th>$F$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthmatic group</td>
<td>8</td>
<td>0.85 ± 0.07</td>
<td>0.84 ± 0.09</td>
<td>0.67 ± 0.10</td>
<td>11.657</td>
<td>0.000</td>
</tr>
<tr>
<td>Control group</td>
<td>8</td>
<td>0.89 ± 0.12</td>
<td>0.95 ± 0.07</td>
<td>0.94 ± 0.10</td>
<td>1.096</td>
<td>0.353</td>
</tr>
</tbody>
</table>

$^1$Compared with control group at 21d, $P<0.05$; $^2$Compared with control group at 34d, $P<0.05$; $^3$Compared with asthmatic group at 21d, $P<0.05$; $^4$Compared with asthmatic group at 34d, $P<0.05$.

Comparison of Erythrocyte Magnesium Levels in Asthmatic Group and Control Group

At day 1, no significant difference was found between the two groups ($t=0.424$, $p=0.678$). At day 21 and day 34, the mean erythrocyte magnesium levels were significantly lower in asthmatic group than in control group ($t=4.78, 6.421$, respectively, $p<0.05$). For the control group, there was no significant difference in erythrocyte magnesium concentrations at day 1, day 21 and day 34 ($F=0.616, p=0.55$). For the asthmatic group, significant difference in erythrocyte magnesium concentrations were found among day 1, day 21 and day 34 ($F=12.52, p=0.000$): the erythrocyte magnesium concentrations at day 34 were significantly lower than that of day 1 and day 21 ($q=4.87, 3.41$, respectively, $p<0.05$) (Table II).

Table II. Comparison of erythrocyte Mg$^{2+}$ between asthmatic group and control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>1d (mmol/L)</th>
<th>21d (mmol/L)</th>
<th>34d (mmol/L)</th>
<th>$F$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthmatic group</td>
<td>8</td>
<td>2.48 ± 0.14</td>
<td>2.39 ± 0.14</td>
<td>2.17 ± 0.08</td>
<td>12.52</td>
<td>0.000</td>
</tr>
<tr>
<td>Control group</td>
<td>8</td>
<td>2.49 ± 0.07</td>
<td>2.44 ± 0.09</td>
<td>2.43 ± 0.08</td>
<td>0.616</td>
<td>0.55</td>
</tr>
</tbody>
</table>

$^1$Compared with control group at 21d, $P<0.05$; $^2$Compared with control group at 34d, $P<0.05$; $^3$Compared with asthmatic group at 21d, $P<0.05$; $^4$Compared with asthmatic group at 34d, $P<0.05$.

Renal mRNA Expression of TRPM6 by RT-PCR

RT-PCR showed that the renal TRPM6 mRNA expression declined significantly in asthmatic group than the control group. Band density was analyzed by software (see Materials and Methods), and values were expressed as the proportional relations between the density of renal TRPM6 mRNA expression and that of actin. At day 1, there was no significant difference in the TRPM6 mRNA expression between the two groups ($t=0.54$, $p=0.597$). At day 21 and day 34, the TRPM6 mRNA expression was significantly lower in asthmatic group than in control group ($t=7.402, 10.526$, respectively, $p<0.05$). For the control group, there was no significant difference in the TRPM6 mRNA expression at day 1, day 21 and day 34 ($F=0.616, p=0.55$). For the asthmatic group, significant difference in plasma magnesium concentrations were found among day 1, day 21 and day 34 ($F=0.616, p=0.55$). The erythrocyte magnesium concentrations at day 34 were significantly lower than that of day 1 and day 21 ($q=4.87, 3.41$, respectively, $p<0.05$) (Table II).
21 and day 34 ($F=0.983$, $p=0.391$). For the asthmatic group, the TRPM6 mRNA expression at day 21 and day 34 were significantly lower than that at day 1 ($q=5.347, 8.201$, respectively, $p<0.05$) and the TRPM6 mRNA expression at day 34 was significantly lower than that at day 21 ($q=3.206, p<0.05$) (Figure 1 and Table III).

**Correlation Between Renal TRPM6 mRNA Expression and Plasma as well as Erythrocyte Magnesium Levels**

Both plasma Mg$^{2+}$ and intracellular Mg$^{2+}$ were positively correlated with TRPM6 mRNA expression in the renal tissues ($r=0.630, 0.715$; $P<0.01$ respectively).

**Discussion**

Our study investigated the relationship between renal TRPM6 mRNA expression and hypomagnesemia in C57BL/6 asthmatic mice. The decreased renal TRPM6 mRNA expression was positively correlated with reduced plasma and erythrocyte magnesium levels, suggesting a possible role of TRPM6 in the status of magnesium deficiency in asthma.

The TRPM6 channel belongs to the TRPM subfamily and is specifically localized along the apical membrane of kidney and small intestine$^{14}$. A few studies have demonstrated that TRPM6 is involved in active epithelial magnesium transport and forms the gatekeeper of magnesium reabsorption in the kidney$^{15,16}$. Walder and Schlingmann reported that TRPM6 mutation caused hypomagnesemia with secondary hypocalcemia$^{9,16,17}$. Rondon et al$^{10}$ reported that downregulation of TRPM6 in the kidney of inulin fed mice could be related to reduced Mg$^{2+}$ reabsorption. Our study showed that decreased renal TRPM6 mRNA expression was significantly associated with reduced plasma and erythrocyte magnesium levels, which indicated that reduced expression of renal TRPM6 might result in hypomagnesemia in asthma.

In this study we found the renal TRPM6 mRNA expression declined significantly in asthmatic group than the control group. Although TRPM6 may be regulated by many factors including dietary magnesium, magnesiotropic hormones and drugs, the regulatory mechanism of TRPM6 expression remains unclear$^{18}$. Epidermal growth factor(EGF) was identified as a hormonal regulator of TRPM6 activity$^{19}$, and it can up-reg-

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**Table III.** Comparison of TRPM6 mRNA of renal tissue between asthmatic group and control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>1d</th>
<th>21d</th>
<th>34d</th>
<th>$F$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthmatic group</td>
<td>8</td>
<td>0.51 ± 0.08$^{4}$</td>
<td>0.32 ± 0.06$^{4}$</td>
<td>0.24 ± 0.05$^{2,3}$</td>
<td>38.176</td>
<td>0.000</td>
</tr>
<tr>
<td>Control group</td>
<td>8</td>
<td>0.49 ± 0.06</td>
<td>0.52 ± 0.05</td>
<td>0.52 ± 0.05</td>
<td>0.983</td>
<td>0.391</td>
</tr>
</tbody>
</table>

$^1$Compared with control group at 21d, $P<0.05$; $^2$Compared with control group at 34d, $P<0.05$; $^3$Compared with asthmatic group at 21d, $P<0.05$; $^4$Compared with asthmatic group at 34d, $P<0.05$.  

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Figure 1. Expression of TRPM6 mRNA in renal tissues of asthmatic mice. M: DNA ladder marker; 1: control group at 1d; 2: asthmatic group at 1d; 3: control group at 21d; 4: asthmatic group at 21d; 5: control group at 34d; 6: asthmatic group at 34d.
ulate TRPM6 expression and magnesium influx by increased phosphorylation of ERK1/2. Meanwhile, ischemia and hypoxia of kidney may be caused by airway obstruction and imbalance of ventilation/perfusion ratio in acute attack of severe asthma, while the expression of EGF was decreased in kidneys with ischemia/reperfusion injury. Therefore, it is likely that reduced EGF levels may lead to decreased TRPM6 expression in asthma.

To date there is extensive research on the magnesium status in asthma patients, but the results remain controversial. Our research showed that the concentration of plasma and erythrocyte magnesium declined significantly in asthmatic group than the control group, which is in accordance with most of previous research. In a prospective study among ninety-three chronic stable asthmatic patients, the prevalence of hypomagnesaemia was 27% and hypomagnesaemia was associated with severity of asthma. Hashimoto et al reported that 40% of asthmatic patients demonstrated magnesium deficiency and magnesium concentration in erythrocytes were significantly decreased in asthma patients. However, other researches failed to find a correlation between hypomagnesemia and asthma, and this we think may due to different methods for magnesium detection, severity of enrolled patients, the effects of treatment and so on.

In conclusion, decreased renal TRPM6 mRNA expression was found in C57BL/6 asthmatic mice, and was positively correlated with reduced plasma and erythrocyte magnesium levels. These findings suggest that TRPM6 may play a role in the in the hypomagnesemia status in asthma.

Acknowledgements

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References


