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ORIGINAL CONTRIBUTION

Erythrocyte magnesium fluxes in mice with nutritionally and genetically low magnesium status

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Summary Low intracellular magnesium (Mg) contents may be observed in case of severe Mg insufficient intake or because of genetic regulation. This work was conducted to investigate the influence of intracellular Mg content on erythrocyte Mg^{2+} influx and efflux in mice with low nutritionally and genetically (MGL and MGH mice) Mg status. C57BL6 mice were fed for 2 wks a diet containing 1000 mg Mg/kg diet Mg (control group), 100 mg Mg/kg diet (Mg-marginal group) or 30 mg Mg/kg diet (Mg-deficient group), while mice with low (MGL) and high (MGH) Mg levels were fed a control diet for 2 wks. The quantification of erythrocyte Mg^{2+} influx and efflux was performed using a stable isotope of Mg. Our results showed that erythrocyte Mg^{2+} influx and efflux were respectively increased and decreased in nutritional Mg deficiency; while in genetically determined Mg status Mg^{2+} fluxes were lower in MGL mice compared to MGH mice. Moreover Mg^{2+} efflux was significantly correlated to Mg level in erythrocytes in all the mice studied ($p < 0.001$). In conclusion, erythrocyte Mg^{2+} influx and efflux are modulated by low Mg status, namely decreased Mg^{2+} efflux compensate for nutritional Mg deficiency, while the genetic regulation of erythrocyte Mg^{2+} content depends on modification of Mg^{2+} influx.

Key words Mg^{2+} influx – Mg^{2+} efflux – erythrocyte – stable isotope – Mg status – genetic regulation

Introduction

Magnesium (Mg), the second most common intracellular cation, is involved in many enzymatic reactions and is critical in ion transport systems [1]. Intracellular free Mg is maintained at a relatively constant level, even if the extracellular Mg level varies, due to specific Mg^{2+} transport systems that regulate the rates at which Mg^{2+} is taken up into cells or extruded from cells. Mg^{2+} influx is basically driven by the electrochemical gradient via channels and carriers [2]; recently TRPM7, a candidate for the regulation of Mg^{2+} influx, has been described and characterised at a molecular and functional level [3]. Cellular Mg output results mainly from a Na^+- and ATP-dependent Mg^{2+} efflux via an Na^+/Mg^{2+} antiport [4, 5].

Despite the regulation of cellular Mg homeostasis, low intracellular Mg content may be observed in case of severe Mg insufficient intake, or because of dysregulation of Mg metabolism. Moreover, cell Mg levels may be modulated by genetic factors [6].

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Until recently, studies on Mg^{2+} flux were limited by the fact that simultaneous determination of Mg^{2+} flux was not possible. In our laboratory, we have developed a new method using a stable isotope of Mg, which allows the simultaneous determination of Mg^{2+} influx and efflux in erythrocytes [7, 8]. We showed that modifying extracellular Mg concentrations, to mimic hypo-magnesaemia, Mg^{2+} efflux and Mg^{2+} influx increased with extracellular Mg up to 0.8 mM, which corresponds to the physiologic concentration of total extracellular Mg.

In this work, we investigated the influence of intracellular Mg content on erythrocyte Mg^{2+} flux in two mice models presenting low Mg status: nutritionally Mg-deficient mice and genetically low Mg mice. The latter model of mice selected for low (MGL) and high (MGH) Mg levels was developed to investigate the mechanisms of the genetic regulation of cellular Mg homeostasis [9, 10].

Materials and methods

Animals and diets

Sixty female C57BL6 mice (Charles River, L'Arbresle, France) aged 18 weeks and forty female mice selected for high (MGH) and low (MGL) erythrocyte Mg levels, aged 17 weeks, were studied. MGL and MGH mice, developed by Henrotte et al. [9], were bred in our laboratory animal colony (National Institute of Agronomic Research, Clermont-Ferrand-Theix, France). These mice were obtained from a bidirectional selective breeding over 18 generations from a heterogenous outbred population of F2 segregant hybrids among the four inbred strain C57BL6, DBA2, C3He and AKR. Assortive mating of mice with the lowest erythrocyte Mg values produced the MGL strain, and assortive mating of mice with the highest erythrocyte Mg values produced the MGH strain [11]. Mice were housed under conditions of constant temperature (20–22 °C), humidity (45–50 %) and a standard dark cycle (20.00–08.00 hours). Our institution guidelines for the care and use of laboratory animals were observed.

C57BL6 mice were randomised into three groups of 20 animals; for 2 wk, each was fed a semipurified diet containing 1000 mg Mg/kg diet Mg (control group), 100 mg Mg/kg diet (Mg-marginal deficient group) or 30 mg Mg/kg diet (severe Mg-deficient group). MGL and MGH mice received a control diet (1000 mg Mg/kg in their diet) for 2 wk. All mice were given free access to distilled water and food. The semipurified diets contained the following (g/kg): casein 200, starch 650, corn oil 50, alphacel (cellulose) 50, DL-methionine 3, choline bitartrate 2, modified AIN-93 mineral mix 35, AIN-97A vitamin mix 10 (ICN Biomedicals, Orsay, France) [12]. Mg level in the three experimental diets was determined by flame atomic absorption spectrometric analysis (Perkin Elmer 800, St-Quentin-en-Yvelines, France).

Evaluation of Mg status

Blood was obtained from anaesthetised mice with pentobarbital (Ceva Santé Animale, Libourne, Fr) by punction in the abdominal vein with a heparinised syringe (sodium heparinate, Panpharma SA Fougères, Fr). Tibia were also collected. A blood counter (Animal Blood Counter, Strasbourg, France) was used to determine hematological parameters for C57BL6 mice. In order to obtain enough red blood cells for Mg flux measurement, blood from 2 mice were pooled. Samples were then centrifuged at 1000 g for 10 min at 20 °C and RBC were safeguarded. For total Mg determination, plasma and haemolyzed erythrocytes were diluted in 0.1 % lanthanum chloride. For tibia analysis, the tissue was first dried, dry-ashed for 10 hours at 500 °C, taken up with HNO_3 and H_2O_2 and heated at 110 °C, and then dried down. The dry residue was taken up with concentrated HNO_3 and appropriately diluted in 0.1 % lanthanum chloride. Mg concentration was determined by atomic absorption spectrophotometry (Perkin Elmer 800, St-Quentin-en-Yvelines, France) at 285 nm.

Mg^{2+} efflux and Mg^{2+} influx [7]

The erythrocytes were washed twice at 20 °C in 10 ml of the washing solution (KCl 140 mmol/L, Hepes 30 mmol/L pH 7.4, sucrose 50 mmol/L, glucose 5 mmol/L), suspended at 10 % in the incubation media (NaCl 150 mmol/L, Hepes 10 mmol/L pH 7.4, glucose 5 mmol/L, ^{25}Mg 0.4 mmol/L) and were incubated at 37 °C. Aliquots of the cell suspension (1 mL) were centrifuged (5000 g, 5 min, 20 °C) at the beginning of the incubation [0] and after 120 min (T). The supernatant was separated, and erythrocytes were washed twice with the washing solution and haemolised for Mg analysis.

^{24}Mg, ^{25}Mg and ^{26}Mg content in supernatants and erythrocytes was determined by ICP/MS (Elan 6100 DRC, PERKIN ELMER, Courtaboeuf, France) after appropriate dilution with 0.14 M HNO_3 using beryllium (Be) and Mg as internal and external standards, respectively. Erythrocyte Mg^{2+} efflux and influx were then calculated according to the following equations:

- Mg^{2+} efflux in µmol/L cells = $[^{24}Mg]_T - [^{24}Mg]_{T0}$ in supernatant × erythrocyte dilution in the cell suspension
- Mg^{2+} influx in µmol/L cells = $[^{25}Mg$ in cells – ^{26}Mg in cells$]_T \times 0.1 - [^{25}Mg$ in cells – ^{26}Mg in cells$]_{T0} \times 0.1$, where 0.1 is the natural abundance of ^{25}Mg.

Given the calibration process, the ICP-MS machine gives

the quantitative results of each measured isotope as if the natural abundance for each isotope was 100 %. Therefore, it was necessary to subtract the amount of ^{26}Mg from the amount of ^{25}Mg in the erythrocyte. Indeed, erythrocyte ^{26}Mg represents the natural erythrocyte Mg but erythrocyte ^{25}Mg represents both the natural erythrocyte Mg plus ^{25}Mg derived from the incubation medium.

To exclude Mg^{2+} efflux caused by cell damage, erythrocyte haemolysis was systematically measured by haemoglobin (Hb) determination in the supernatants (cyanmethaemoglobin, at 546 nm). An haemolysis in supernatants lower than 1.5 % of total haemolysis, which did not increase significantly during the experiment, was considered in the range of normality and was therefore not taken into account.

^{25}Mg enrichment

^{25}Mg enrichment in NaCl medium was measured in erythrocytes concomitantly to Mg flux measurement, as previously described in whole blood [13].

Statistical analysis

Results were expressed as means ± SD. Statistical analysis were based on Student's t-test, on one-way ANOVA followed by a Tukey Kramer multiple comparisons test and on simple linear regression. The limit of statistical significance was set at $P < 0.05$. Statistical analyses were performed using the GraphPad program (V3.00, GraphPad Software, San Diego, CA).

Results

Parameters of the mice and Mg status

The weight of mice fed the Mg-deficient diet was significantly less than that of those fed the control diet or the marginally deficient diet (Table 1). For Mg-deficient diet group, only 4 pooled blood samples were analysed because of the mortality during the experiment. Mg content in plasma, erythrocytes and tibia were significantly lower in the Mg-deficient group compared to the control group or the marginally Mg-deficient group. No difference was observed between the control group and the marginally Mg-deficient group for the studied parameters.

The weight of MGH mice was significantly lower than for MGL mice (Table 2). Mg content in plasma, erythrocytes and tibia were significantly lower in MGL mice compared to MGH mice (Table 2).

Haematological parameters in the controls, marginally Mg-deficient or Mg-deficient mice

Red blood cell (RBC) counts were significantly increased, while Hb and haematocrit (Ht) levels and mean corpuscular volume (MCV) were significantly decreased in Mg-deficient mice by comparison to control mice and marginally Mg-deficient mice (Table 3), reflecting anemia in Mg-deficient mice.

Erythrocyte Mg^{2+} influx and Mg^{2+} efflux

As shown in Fig. 1, erythrocyte Mg^{2+} influx was significantly increased in mice when Mg level in the diet was decreased ($p < 0.05$). Mg^{2+} influx increased from 6 % in marginal Mg-deficient diet to 14 % in severe Mg-defi-

Table 2 Characteristics of the MGH and MGL mice

	MGH	MGL
Weight[1] (g)	25.9 ± 4.8	30.2 ± 2.8*
Plasma Mg (mmol/L)	1.09 ± 0.10	0.94 ± 0.06*
Erythrocyte Mg (mmol/L)	2.69 ± 0.25	2.14 ± 0.21**
Tibia Mg (mmol/g dry tibia)	0.249 ± 0.018	0.154 ± 0.019**

Results are mean ± SD, n = 10 except for tibia MGL where n = 8; [1] n = 20
Statistical significance: * $p < 0.01$, ** $p < 0.001$

Table 1 Characteristics of the controls, marginally Mg-deficient or Mg-deficient mice

Characteristics	Control mice (1000 mg Mg/kg diet)	Marginally deficient (100 mg Mg/kg diet)	Deficient mice (30 mg Mg/kg diet)
Weight[1] (g)	20.5 ± 0.8[a]	20.6 ± 1.1[a]	19.5 ± 0.7[b]
Plasma Mg (mmol/L)	0.856 ± 0.126[a]	0.845 ± 0.060[a]	0.215 ± 0.044[b]
Erythrocyte Mg (mmol/L)	2.43 ± 0.16[a]	2.21 ± 0.38[a]	0.96 ± 0.13[b]
Tibia Mg (mmol/g wet tibia)	0.107 ± 0.009[a]	ND	0.080 ± 0.013[b]

Results are mean ± SD, n = 10 except for deficient mice where n = 4 for plasma and erythrocytes
[1] n = 20 except for deficient mice where n = 8. Means in a row not sharing the same superscript are significantly different (One way-ANOVA followed by a Tukey Kramer multiple comparisons test, $p < 0.05$)
ND not determined

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Table 3 Haematological parameters of the controls, marginally Mg-deficient or Mg-deficient mice

	Control mice (1000 mg Mg/kg diet)	Marginally deficient (100 mg Mg/kg diet)	Deficient mice (30 mg Mg/kg diet)
RBC (10^6/mm^3)	7.45±0.66[a]	7.61±0.65[a]	8.65±0.50[b]
MCV (μm^3)	43.6±0.60[a]	43.6±0.60[a]	41.8±0.67[b]
Hb (g/dL)	12.72±1.10[a]	12.99±1.11[a]	14.34±0.91[b]
Ht (%)	32.47±2.91[a]	33.14±2.78[a]	36.27±2.27[b]

Results are mean ± SD, n = 20 except for deficient mice where n = 8. Means in a row not sharing the same superscript are significantly different (One way-ANOVA followed by a Tukey Kramer multiple comparisons test, $p < 0.05$)
RBC red blood cells; *MCV* mean corpuscular volume; *Hb* haemoglobin; *Ht* haematocrits

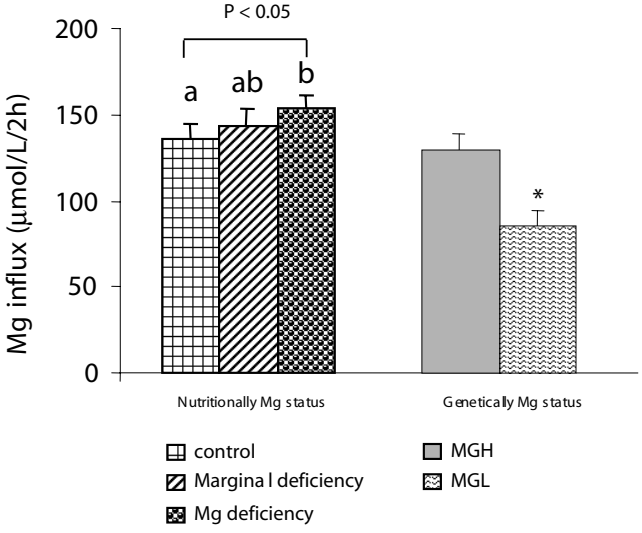

Fig. 1 Mg influx in erythrocytes from control, marginally Mg-deficient and Mg-deficient mice and in erythrocytes from MGH and MGL mice. Results are mean ± SD, n = 10 except for deficient mice where n = 4. For mice with nutritionally determined Mg status, means not sharing the same superscript are significantly different (One way-ANOVA followed by a Tukey Kramer multiple comparisons test, $p < 0.05$) and for mice with genetically determined Mg status, statistical significance (student t test): * $p < 0.001$

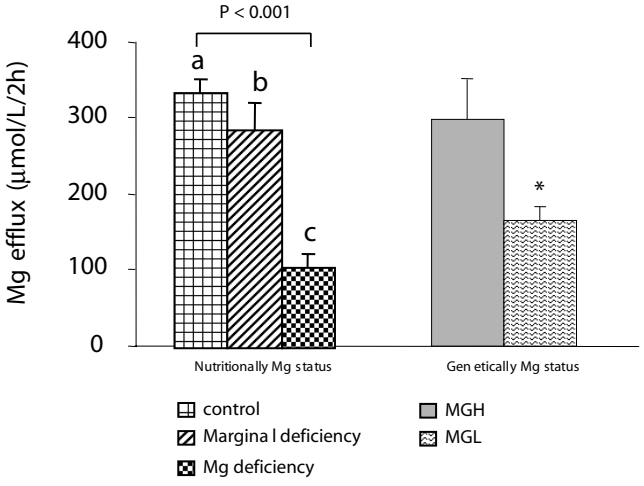

Fig. 2 Mg efflux in erythrocytes from control, marginally Mg-deficient and Mg-deficient mice and in erythrocytes from MGH and MGL mice. Results are mean ± SD, n = 10 except for deficient mice where n = 4. For mice with nutritionally determined Mg status, means not sharing the same superscript are significantly different (One way-ANOVA followed by a Tukey Kramer multiple comparisons test, $p < 0.05$) and for mice with genetically determined Mg status, statistical significance (student t test): * $p < 0.001$

cient diet. Moreover, as shown in Fig. 2, erythrocyte Mg^{2+} efflux was significantly decreased when the Mg level in the diet was decreased ($p < 0.001$). It decreased from 15 % in marginally Mg-deficient diet to 70 % in severely Mg-deficient diet.

As shown in Figs. 1 and 2, erythrocyte Mg^{2+} influx and efflux were significantly lower in MGL mice compared to MGH mice ($p < 0.001$). Mg^{2+} influx and efflux were respectively 38 % and 45 % lower in MGL mice than in MGH mice.

Fig. 3 showed that Mg^{2+} efflux was significantly correlated to Mg level in erythrocytes in all the studied mice ($p < 0.001$), and that Mg^{2+} influx was not correlated to erythrocyte Mg level ($p = 0.7626$). It is interesting to note that these results are not influenced by the possible genetic control of MGL and MGH mice, as Mg^{2+} efflux was still significantly correlated to Mg level ($p < 0.001$) and

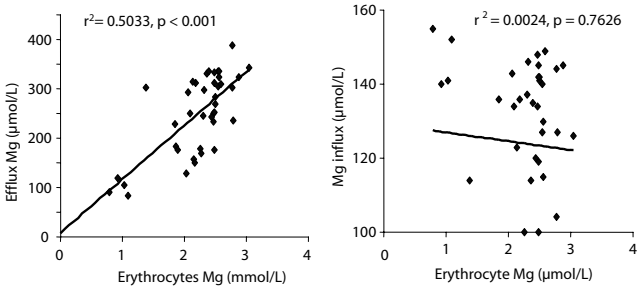

Fig. 3 Correlation between erythrocyte Mg level and Mg efflux and influx in mice: n = 40, the regression line is 108.8x + 8.31 for Mg efflux, and –2.4135x + 129.41 for Mg influx

Mg^{2+} influx was not correlated to Mg level ($p = 0.1779$) when considering only controls, marginally Mg-deficient and Mg-deficient mice.

^{25}Mg enrichment

^{25}Mg enrichment in percent was significantly higher in erythrocytes from marginally Mg-deficient mice compared to control mice, and in erythrocytes from severely Mg-deficient mice compared to marginally Mg-deficient mice (Table 3). ^{25}Mg enrichment was two-fold increased in erythrocytes from Mg-deficient mice compared to marginally Mg-deficient mice and control mice.

^{25}Mg enrichment in percent was significantly lower in erythrocytes from MGL mice compared to MGH mice (Table 4). ^{25}Mg enrichment was 30 % lower in erythrocytes from MGL mice compared to MGH mice.

Discussion

As expected during Mg deficiency, markers of Mg status were altered in Mg-deficient mice by comparison to controls. In fact, plasma, erythrocyte and tibia Mg concentrations decreased considerably in Mg-deficient mice. On the other hand, plasma and erythrocyte concentrations were not different from control levels in marginally Mg-deficient mice. This observation was not unexpected and reflected the well known limitation of plasma and erythrocyte Mg levels as biochemical markers of Mg status [14]. Since serum Mg represents < 1 % of total body Mg content, the serum Mg concentration may not reflect intracellular Mg status. Moreover, erythrocyte Mg concentration is not correlated with other tissue pools of Mg [15], as an apparent genetic regulation of erythrocyte Mg may be a confounding factor accounting for this lack of correlation [6]. Tibia, in turn, is an excellent tissue marker of Mg status, but unfortunately tibia Mg content was not measured in marginally Mg-deficient mice because of a technical problem in processing this tissue. Severely Mg-deficient mice presented anaemia, with more red blood cells but lower mean corpuscular volume and haemoglobin content, by comparison to control or marginally Mg-deficient mice, as previously described in rats [16].

Table 4 ^{25}Mg enrichment in erythrocytes from the studied mice

	^{25}Mg enrichment (%)
Control mice (1000 mg Mg/kg diet)	59.9 ± 2.0^{a}
Marginally deficient (100 mg Mg/kg diet)	64.4 ± 1.6^{b}
Deficient mice (30 mg Mg/kg diet)	130 ± 7^{c}
MGH mice (1000 mg Mg/kg diet)	72.4 ± 3.3
MGL mice (1000 mg Mg/kg diet)	51.3 ± 3.9*

Results are mean ± SD, n = 10 except for deficient mice where n = 4
Statistical significance: means for control, marginally Mg-deficient or Mg-deficient mice not sharing the same superscript are significantly different (One way-ANOVA followed by a Tukey Kramer multiple comparisons test, $p < 0.001$); * $p < 0.001$ between MGH and MGL mice

Exploration of Mg^{2+} fluxes during Mg deficiency showed that Mg^{2+} fluxes were altered during both severely and marginally Mg deficiency. Mg^{2+} influx increased with the intensity of Mg deficiency, while Mg^{2+} efflux decreased. As during marginally Mg-deficiency, erythrocyte Mg content was not modified, we can assume that the modifications of Mg^{2+} fluxes were sufficient to maintain intracellular Mg homeostasis. In contrast, during severe Mg deficiency, the increase in Mg^{2+} influx and the drastic decrease in Mg^{2+} efflux were not sufficient to prevent Mg depletion in erythrocyte. Our results concerning Mg^{2+} efflux are different from those described by Gunther et al. in Mg-deficient rats [17]. These authors observed that net Mg^{2+} efflux was increased in erythrocytes from Mg-deficient rats. In considering these discrepancies it must be pointed out the technical differences in the animal treatment and in protocols to measure Mg fluxes. In Gunther's study, Mg^{2+} efflux was determined in erythrocytes from rats after 1 month of Mg-deficient diet, and Mg^{2+} efflux was measured by atomic absorption spectrometry after erythrocyte Mg loading, utilising an ionophore that may have altered the erythrocyte membrane. Thus, it seems that in Gunther's study, Mg efflux could be biased by Mg leak from membranes. In support of this interpretation, we previously demonstrated that during Mg deficiency, disturbances in lipid metabolism increased fluidity of the erythrocyte membrane [18]. Our results of the increased Mg^{2+} influx and decreased Mg^{2+} efflux during Mg deficiency are supported by the determination of erythrocyte ^{25}Mg enrichment. The determination of ^{25}Mg enrichment in blood cells was developed in our laboratory as a new biomarker of Mg status [19]. This *in vitro* blood load test was based on the hypothesis of an increase cellular demand for Mg during Mg deficiency, leading to an increased *in vitro* cellular uptake of isotopic Mg. In fact, we demonstrated that ^{25}Mg enrichment in blood cells from Mg-deficient rats was greater than those from controls. In the present study, the *in vitro* load test was not performed in blood but in NaCl medium, and we observed that ^{25}Mg enrichment of erythrocytes from Mg-deficient mice increased with the severity of Mg deficiency. Our results on Mg^{2+} fluxes demonstrated that the higher ^{25}Mg enrichment during Mg deficiency resulted both from an increased in Mg^{2+} influx and a decreased in Mg^{2+} efflux. These data however do not rule out the mechanisms of intracellular Mg regulation: raising the question whether Mg^{2+} efflux counteracts Mg^{2+} influx or vice versa, or whether both fluxes regulates cellular Mg homeostasis.

We also investigated Mg^{2+} fluxes in mice selected for low Mg levels (MGL) and high Mg levels (MGL). MGL and MGH mice were specifically developed by Henrotte to investigate the mechanisms and biological significance of the genetic regulation of blood Mg levels [9]. We previously demonstrated that MGL mice presented

lower body stores of Mg and lower body retention of Mg, and in particular, smaller total Mg exchangeable pool masses [10]. As expected, MGL mice presented lower plasma, erythrocytes and tibia Mg levels compared with MGH mice. Moreover the weight of MGH mice was significantly lower than for MGL mice. This has been previously observed by us (unpublished observation), although it is in contrast with Henrotte's observation of slightly but not significantly lower body weight in MGL mice than in MGH mice [9]. When exploring Mg^{2+} fluxes, we observed lower Mg^{2+} influx and lower Mg^{2+} efflux in MGL mice by comparison to MGH mice. This result may seem in contrast with those previously published by our group [10]. In that work, we observed higher Mg^{2+} efflux in erythrocytes from MGL mice by comparison to MGH mice. Noteworthy, erythrocytes were loaded with Mg prior to Mg^{2+} efflux measurements, according to Gunther and Vormann [20]. In light of the discrepancy between the present and previous results we can affirm that artificial erythrocyte Mg loading can lead to erroneous results concerning physiological Mg^{2+} fluxes. ^{25}Mg enrichment was similar to that previously observed, i. e. lower enrichment of ^{25}Mg in erythrocytes of MGL mice by comparison to MGH mice, despite a lower Mg level, and contrary to what was observed in Mg-deficient mice compared to Mg-sufficient mice [10]. Our present results demonstrated that the lower retention of Mg by MGL erythrocytes in comparison to MGH erythrocytes was not due to enhance Mg^{2+} efflux but rather to a lower Mg^{2+} influx, suggesting a genetic control of erythrocyte Mg homeostasis by a regulation of uptake rather than release of Mg.

When correlating Mg^{2+} efflux to erythrocyte Mg levels for all the studied mice, we observed a positive correlation. This observation suggests that physiological intracellular Mg levels influence Mg^{2+} efflux. This is coherent with the observations of Ebel et al. [21] and Ferreira et al. [22] who reported an increase of Mg^{2+} efflux by Mg loading. In contrast, no correlation was observed between Mg^{2+} influx and erythrocyte Mg content. Recently, Schmitz et al. [23] showed that the activity of TRPM7, a candidate for the regulation of Mg^{2+} influx, declines as intracellular ionised Mg concentration reaches supra-physiological concentrations. In light of these observations, our data demonstrated that with physiological intracellular Mg levels, erythrocyte Mg^{2+} influx, exploited either by TRPM7 or any other channel, was not influenced by the intracellular Mg level.

In conclusion, our results demonstrated that erythrocyte Mg^{2+} influx and efflux are modified with Mg status. We provided evidence to show that the intracellular Mg level may modulate Mg^{2+} efflux and we suggested a genetic regulation of erythrocyte Mg^{2+} influx.

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