Effects of supplementation with different Mg salts in cells: is there a clue?

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Abstract. The differing bioavailability of magnesium salts remains an open question, both at the cellular and systemic level. However, this issue is relevant for identifying the most effective magnesium supplement. We compared the effects of three widely used magnesium salts: MgSO4, MgCl₂ and Mg pidolate, on the proliferation of four human cell types: promyelocytic leukaemia HL60, osteoblast-like Saos-2 and U-2 OS, and endothelial cells from the umbilical vein. The three magnesium salts had no effect on endothelial and leukemic cell growth, but magnesium pidolate impaired cell growth in osteoblast-like cells. In particular, in Saos-2 cells, 1 mM pidolate induced a slight accumulation of cells in the G0/G1 phase of the cell cycle and, in parallel, an early rise in intracellular calcium and a late decrease in intracellular magnesium content. Interestingly, when cultured in 5 mM magnesium pidolate, Saos-2 cells grew as fast as the controls. Moreover, intracellular magnesium and calcium concentrations did not vary. These results suggest a lower bioavailability of magnesium pidolate in osteoblast-like cells.

Key words: magnesium pidolate, intracellular magnesium, intracellular calcium, cell proliferation

Magnesium (Mg) is the fourth most abundant mineral in the human body. About 50% is stored in the bone, while the remaining 50% is predominantly localised inside the cells of body tissues and organs [1]. Only 1% of the Mg is found in blood, where its levels are kept constant mainly by the bone exchangeable pool [2]. Indeed, it has been proposed that serum and bone surface Mg concentrations are closely related, indicating a continuous exchange of Mg between bone and blood [3-5]. This suggests that bone plays an important role as a buffer, protecting against acute changes in serum Mg content, although this has not been studied as extensively as for calcium (Ca) regulation [6]. However, beside the skeleton, the gastrointestinal tract and kidneys are involved in Mg homeostasis, regulating absorption and excretion, under metabolic and hormonal influences [7, 8].

Being the cofactor of several hundreds of enzymes, Mg is involved in all major cellular processes, in primis in energy metabolism, acting as
a counter-ion for ATP and nucleic acids, and being necessary to stabilize enzymes, including those implicated in many ATP-generating reactions [9-11]. Now the pivotal role of Mg in regulating ion channels, metabolic cycles and signalling pathways is widely recognised [11].

An open and quite controversial question concerns the optimum Mg supplement to use, as highlighted by the heated debate recently reported in this journal [12, 13]. Few systematic studies have been published on animals or humans, demonstrating that the different salts currently used in Mg therapeutic formulation are able to supply enough bioavailable Mg, although the neutral formulations coupling this cation to organic acids or to amino acids seem to have the highest bioavailability [14-16]. These salts displayed a very low dissociation constant in physiological conditions (i.e. 37°C and ionic strength of 0.15 mol/L), providing that they can overcome the acidic gastric environment without being ionised and are easily absorbed through the gastrointestinal barrier by a mechanism of diffusion [14, 17, 18]. Mg pidolate represents an important exception among the organic salts, as its dissociation constant is similar to the inorganic salts, being highly dissociated at physiological pH [17, 18]. However, this counter-ion also seems to play an important role in Mg cellular absorption for inorganic salts. Durlach et al. [19] demonstrated differences between the pharmacological and toxicological properties of MgSO₄ and MgCl₂, stressing that absorption and retention are more efficient with MgCl₂ than with MgSO₄.

An additional problem in studying Mg supplementation in whole organisms and, in particular, in humans, is the assessment of Mg status after its administration, because there are no simple, rapid, and accurate laboratory tests to measure total body Mg stores or to evaluate its distribution in the different compartments [16]. At the cellular level, literature on the different salts is even more scarce. It is known that several mammalian cells exposed to low or null Mg do not usually display a significant decrease in intracellular Mg content due to its very slow turnover [11, 20, 21]. However, effects of the extracellular Mg concentration on cellular functions such as proliferation [22, 23] or differentiation [24] have been reported.

In this study, we tested the effects of three widely used Mg salts, i.e. MgSO₄, MgCl₂ and Mg pidolate, on cell proliferation and intracellular content of Mg and Ca, in endothelial cells isolated from the human umbilical vein (HUVEC), and three human cell lines: the promyelocytic leukaemia HL60, the osteoblast-like cells Saos-2 and U-2 OS.

Materials and methods

Reagents

All reagents were obtained from Sigma Aldrich (St. Louis, Mo, USA), if not otherwise specified, and were Ultrapure grade. Dulbecco’s phosphate-buffered saline (DPBS) without Mg (8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L Na₂HPO₄, 0.2 g/L KH₂PO₄, pH 7.2) was prepared in doubly-distilled water. Foetal bovine serum (FBS) was stripped of Mg by dialysis, using a dialysis membrane (Medicell International LTD, UK) with a 3500 D cut-off against Puck buffer plus EDTA 1 mM for two days, and Puck buffer alone for the last three days. The Ca content in dialysed FBS was restored by adding CaCl₂ at a final concentration 1.8 mM. The FBS was then sterilised by filtering through a 0.45 μm filter.

Cell culture

The cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Saos-2, U-2 OS, and HL60 cells were grown in MEM (Invitrogen, San Giuliano M.se, Italy), supplemented with 2 mM L-glutamine, 10% FBS, 1000 units/mL penicillin and 1 mg/mL streptomycin, and is referred as the “control medium” in the following text. HUVEC were isolated from the umbilical vein and cultured in M199 containing 10% FBS, 1 mM glutamine, 1000 units/mL penicillin and 1 mg/mL streptomycin, endothelial cell growth factor (ECGF) 150 μg/mL, 1 mM sodium pyruvate and heparin (5 units/mL) on 2% gelatine-coated dishes. Mg-free MEM was purchased from Invitrogen and utilised to study the effects of different salts of Mg by adding MgSO₄, MgCl₂, or Mg pidolate at 1 or 5 mM. For these media, dialysed FBS was used. We also used medium containing 0.1 mM Mg (MEM 0.1 mM Mg), which was obtained by adding non-dialysed 10% serum to Mg-free MEM as described [22, 24].

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In the first set of experiments, all cell types were seeded in control medium; 16 hours later they were switched to medium containing 1 or 5 mM MgSO₄, MgCl₂ or Mg pidolate. In another set of experiments, Saos-2 and U-2 OS cells were seeded directly in these media. For proliferation assays, the cells were trypsinised, stained with trypan blue solution (0.4%), and the viable cells were counted using a Burker chamber every 24 h for four days.

To determine intracellular Ca and Mg, Saos-2 cells were seeded in 24-well plates and switched after 16 h to media with 1 mM Mg salts or 0.1 mM Mg, for different time periods.

**Cell cycle evaluation by flow cytometry**

Cell cycle analysis was performed according to Nüsse et al. [25]. Briefly, 2 × 10⁶ cells were centrifuged and 1 mL of solution I (0.584 g/L NaCl, 1.139 g/L sodium citrate, 10 mg/L RNase, 0.3 mL/L Nonidet P-40) was added to the cell pellet. After 30 min, 1 mL of solution II (100 mg/L propidium iodide (PI), 0.25 M sucrose, 15 g/L citric acid) was added to complete the fixing and staining. The cell suspension was agitated and kept at 4°C until flow cytometry measurements. Flow cytometric analyses were carried out using an Epics-XL Beckman Coulter equipped with a 15 mW Argon ion laser source, and analysed with ModFit (Verity Software House, USA) software.

**Quantification of total cell Mg by spectrofluorimetric assay**

Total Mg content was assessed on sonicated cell samples using a fluorescent assay based on the new Mg probe DCHQ5, a diaza-crown-hydroxyquinolines derivative [26, 27]. Briefly, Saos-2 cells were detached, washed twice with DPBS and lysed by sonication in DPBS. The samples were diluted in a 1:1 solution of MetOH:MOPS (2 mM, pH 7.4), stained with DCHQ5 15 μM and analysed using a PTI Quanta Master C60/2000 spectrofluorimeter (Photon Technology International, Inc., NJ, USA) with the excitation wavelength at 360 nm and emission at 510 nm. The Mg concentration of the samples was obtained by interpolation of their fluorescence with a standard curve of MgSO₄ ranging between 0.5 and 6 μM.

**Results**

**Effects of different Mg salts on cell proliferation**

It is widely accepted that Mg is required for cell proliferation in various cells, and that changes in its extracellular concentration modulate their proliferation [29-33]. We therefore evaluated whether different Mg salts affected the growth of different cells. Initially we performed experiments on Saos-2 cells, which were switched to medium containing 1 mM MgSO₄, MgCl₂ or Mg pidolate 16 h after seeding in their own control media. The cells were counted every 24 h for four days. Figure 1 shows that growth in Saos-2 cells was markedly inhibited in medium containing 1 mM Mg pidolate. It must be underlined that pidolic acid alone did not elicit any effect (data not shown), indicating that it is the Mg pidolate that is affecting these cells. When exposed to higher concentration of Mg pidolate, i.e. 5 mM, normal growth rate was restored. To understand whether this puzzling result can be generalised to other cell types, we tested HUVEC, which grow after adhering to extracellular matrix, HL60 cells, which grow in suspension, and U-2

**Measurement of intracellular free Ca with Fura-2/AM**

Intracellular free Ca was evaluated using Fura-2/AM staining according to Malgaroli et al. [28]. Briefly, Saos-2 cells seeded in 24-well plates were incubated with Fura-2/AM 10 μM for 60 min in culture medium. The cells were washed with a buffer containing NaCl 125 mM, KCl 5 mM, MgSO₄ 1.2 mM, CaCl₂ 2 mM, glucose 6 mM, Hepes-NaOH buffer 25 mM (pH 7.4), removed by trypsinisation, suspended in the aforementioned buffer and analysed using the spectrofluorimeter with the excitation wavelength at 360 nm and emission at 450 nm.

**Statistical analysis**

The experiments were repeated at least three times, and statistical significance was determined using the paired Student’s t-test and the one-way ANOVA followed by the Tukey post hoc test. Significance was set at p values less than 0.05 and is reported in the figures with asterisk *.
Figure 1. Growth curves of Saos-2, HL60, HUVEC, and U-2 OS cells cultured in the presence of MgSO4 1 mM (■) and 5 mM (▲), MgCl2 1 mM (▼) and 5 mM (▲), or Mg pidolate 1 mM (●) and 5 mM (●).

OS, which is another osteoblast-like cell line. The presence of the different salts did not significantly affect the proliferation rate of these cells, as confirmed by the statistical analysis performed using Student’s t-test comparing each sample with controls. However, it should be noted that 1 mM Mg pidolate slightly and reproducibly reduced U2-OS cell growth, although statistical significance was not reached. As previously demonstrated, HUVEC cells grew faster in 5 than in 1 mM Mg with all the salts tested [34].

In a second set of experiments, the cells were seeded directly into media containing MgSO4, MgCl2 or Mg pidolate. In agreement with the aforementioned results, MgSO4, MgCl2 or Mg pidolate had similar effects on cell growth in HUVEC and HL60 (data not shown), while Mg pidolate (1 mM) was confirmed to exert an...
Effects of Mg salts in cells

Figure 2. A) Growth rate of Saos-2 cells and B) cell cycle distribution after 72 hours of culture in control medium, in MEM 0.1 mM Mg and MEM containing Mg pidolate 1 and 5 mM. Data were analysed using one way ANOVA followed by Tukey’s test; *, p<0.05 versus control.

inhibitory effect on Saos-2 cells (figure 2A). Again, some slight effect of Mg pidolate at 1 mM was also found on U-2 OS cells. In Saos-2 cells, the impaired proliferation is associated with a slight but significant increment of cells in the G0/G1 phase (figure 2B). Growth rate and cell cycle distribution were comparable to controls when Saos-2 cells were cultured in the presence of 5 mM Mg pidolate, indicating that higher concentrations of extracellular Mg pidolate are necessary to maintain normal cell growth. It is noteworthy that Saos-2 cells showed the same behaviour if cultured in 1 mM Mg pidolate or in 0.1 mM extracellular Mg.

Effect of Mg pidolate on intracellular Ca2+ and Mg in Saos-2 cells

An altered Ca/Mg ratio might be responsible for the growth inhibition seen in Saos-2 cells cultured in the presence of 1 mM Mg pidolate. We therefore measured the intracellular content of Ca2+ and total Mg, both crucially contributing to the regulation of cell growth.

Saos-2 cells grown for 16 h in control medium were switched to media containing 1 mM MgSO4, or MgCl2 or Mg pidolate for 3 and 24 h. We also exposed some samples to medium with 0.1 mM Mg. Under all the experimental conditions we found higher amounts of intracellular Ca2+ after 3 h than after 24 h. In particular, the cells cultured in 1 mM Mg pidolate showed a significant increase in intracellular Ca2+, both at 3 and 24 h if compared to cells grown in control medium or in 1 mM MgSO4- or MgCl2-containing media (figure 3A). Interestingly, the Ca2+ increase was higher in Saos-2 cells exposed to 1 mM Mg pidolate than to medium containing 0.1 mM Mg. It should be noted that pidolic acid alone did not elicit any effect on Ca2+ levels in Saos-2 cells (data not shown). No effect on Mg content was observed at 3 or 24 h under any of the conditions tested (figure 3B). However, after 72 h Saos-2 cells in 1 mM Mg pidolate, but not those in 5 mM Mg pidolate, showed a decrease in total intracellular Mg comparable to that observed in Saos-2 grown in 0.1 mM Mg (figure 4).

Discussion

Mg supplementation is claimed to have beneficial effects both in prevention and in the treatment of several common acute or chronic diseases, as stroke, hypertension, osteoporosis or diabetes, as
well as in less severe conditions such as insomnia, fatigue or headache [35, 36]. Several Mg salts, alone or in combinations have been proposed for use, but exhaustive studies are scarce, both at the systemic and at the cellular level. In this work, we examine three Mg salts that are widely used in oral commercial formulations, i.e. MgCl₂, MgSO₄ and Mg pidolate. They were chosen because they are entirely comparable in their biological effects, being all dissociated at a pH of around 7 [18].

We tested these salts on different human cells. Bone cells were chosen because of the key role of Mg in bone health, leukemic HL60 as a model of cells growing in suspension, and HUVEC, as a model of primary cells growing after adhering to extracellular matrix. The salts were tested at two different concentrations. 1 mM represents the physiological Mg levels, while 5 mM is a high concentration that has been previously used in in vitro studies [37-39].

The effects of MgCl₂, MgSO₄ and Mg pidolate on cell growth were comparable in HUVEC and HL60 cells, while in bone cell lines Mg pidolate effects were significant in Saos-2, and moderate, although not statistically significant, in U-2 OS. In particular, 1 mM Mg pidolate impaired the growth of Saos-2 cells in parallel with a slight accumulation of cells in the G0/G1 phase of the cell cycle. It is noteworthy that no cytotoxicity was observed, at least up to day 7 of exposure to 1 mM Mg pidolate (data not shown). Growth retardation was associated with an altered Ca/Mg balance. Indeed, an increase in intracellular Ca was detected after 3 and 24 h, while a decrease in intracellular Mg became statistically significant after 72 hours. It should be noted that both intracellular Mg levels and proliferation were similar to those found in controls when we culture the cells in 5 mM Mg pidolate. This result suggests that higher concentrations of this salt are required to release enough Mg to maintain the homeostasis of intracellular Mg as well as a normal growth rate. Interestingly, the effects of 1 mM Mg pidolate on Saos-2 cells is mimicked by their exposure to very low extracellular Mg, which inhibits cell growth as previously described [40], increases intracellular Ca and decreases intracellular Mg.
that in Saos-2 cells, low extracellular Mg, as well as 1 mM Mg pidolate, alter the Ca-to-Mg ratio, and that this imbalance is responsible for growth impairment. Our hypothesis is in agreement with Abed and Moreau who stressed that “part of the progression of cell proliferation is ensured by both ions acting, probably together via a common mechanism, but distinct mechanisms are revealed for each ion to reach optimal cell proliferation” [41]. Pointing our attention to intracellular Mg, it is noteworthy that the decrease in the cation occurs late, after 72 h culture in 1 mM Mg pidolate or in 0.1 mM Mg. We argue that initially the cells can compensate for the reduced availability of extracellular Mg by mobilising the cation from binding sites or intracellular stores. At later times, these mechanisms are not sufficient to overcome the reduced availability of extracellular Mg and, consequently, the cells cannot maintain the intracellular concentration necessary to sustain normal progression in the cell cycle. These results argue in favour of a lower bioavailability of Mg pidolate than other salts in Saos-2 cells. Amazingly, we did not observe any similar effects by adding pidolic acid, thus indicating that these effects are peculiar to Mg pidolate.

This different sensitivity of osteoblast-like cells to Mg pidolate could be due to fact that Saos-2 and U-2 OS possess rather different phenotypes. A first issue to highlight is that both cell types express the channels TRPM6 and 7, which are important for Mg homeostasis [42]. However, more studies are required to define the expression pattern of magnesiotropic channels in these cells since several other channels have been identified in the recent years [43]. In terms of morphology, Saos-2 cells are polygonal and U2-OS are triangle-shaped, but they are the same size. More importantly, Saos-2 cells show a more mature osteoblastic phenotype than U-2 OS. Indeed, while U-2 OS cells are negative for almost all osteoblastic markers, being classified as an intermediate phenotype between osteoblast and fibroblast, Saos-2 cells express osteocalcin, bone sialoprotein, decorin, procollagen I and possess alkaline phosphatase activity [44]. To understand whether the effects of different Mg salts correlate with the degree of cell differentiation, these studies should be broadened to various osteoblast-like cells and, eventually, to mesenchymal stem cells differentiating into osteoblasts. Another striking difference between Saos-2 and U-2 OS regards the expression of the oncosuppressor gene p53. Wild type p53 is expressed and non-mutated in U-2 OS cells, whereas the gene is deleted in Saos-2, an event which explains why Saos-2 cells display a wide range of aneusomy [45]. However, we ruled out a role for the deletion of p53 in mediating the response to Mg pidolate, since p53 is also mutated and inactive in HL60 cells, which are equally sensitive to the three Mg salts. Nevertheless, the explanation of the different behaviour of U-2 OS and Saos-2 cells to Mg pidolate exposure remains elusive.

Mg pidolate however, also shows some peculiarities in other biological contexts. Bac and coll. published in 1995 that this salt, when administered to Mg-deficient rats, has the greatest efficacy because it delays the attack latency and the rate of the interspecific aggressive behaviour compared to other salts (chloride, lactate, aspartate, gluconate) [46]. More recently, Mg pidolate showed good efficacy in the treatment of sickle cell anaemia, since it was able to decrease relative intracellular haemoglobin S concentration by increasing total cell volume via inhibition of normal membrane ion exchange channels, such as the KCl cotransporter and Gardos channels [47].
We conclude that Mg pidolate seems to have a clear, reduced bioavailability in Saos-2 cells, being unable to maintain the normal intracellular Mg content when used at the concentration 1 mM, which corresponds to the normal Mg level in the serum. The effects observed, however, are not due to toxic effects of the salt, because higher doses of Mg pidolate maintain the normal proliferative behaviour of these cells. However more experiments are in progress to clarify the mechanism involved in the peculiar behaviour of magnesium pidolate in bone cells.

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Disclosure

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References


