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Nitric oxide mediates low magnesium inhibition of osteoblast-like cell proliferation☆

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Abstract

An adequate intake of magnesium (Mg) is important for bone cell activity and contributes to the prevention of osteoporosis. Because (a) Mg is mitogenic for osteoblasts and (b) reduction of osteoblast proliferation is detected in osteoporosis, we investigated the influence of different concentrations of extracellular Mg on osteoblast-like SaOS-2 cell behavior. We found that low Mg inhibited SaOS-2 cell proliferation by increasing the release of nitric oxide through the up-regulation of inducible nitric oxide synthase (iNOS). Indeed, both pharmacological inhibition with the iNOS inhibitor L-N^6-(iminoethyl)-lysine-HCl and genetic silencing of iNOS by small interfering RNA restored the normal proliferation rate of the cells.

Because a moderate induction of nitric oxide is sufficient to potentiate bone resorption and a relative deficiency in osteoblast proliferation can result in their inadequate activity, we conclude that maintaining Mg homeostasis is relevant to ensure osteoblast function and, therefore, to prevent osteoporosis.

Keywords: Osteoblast; Nitric oxide; iNOS; Magnesium; Osteoporosis

1. Introduction

Magnesium (Mg) is an essential micronutrient with a wide range of metabolic, structural and regulatory functions [1]. Since it is mainly obtained by eating unprocessed grains and green leafy vegetables [2], Mg inadequacy is rather common in Western countries because of the processing of many foods, the low Mg content in water and soil, and the preference for calorie-rich, nutrient-poor foods [3]. In addition, Mg deficiency occurs in nephropathics, in alcoholics, in patients with diabetes mellitus and in individuals treated with some classes of diuretics or anticancer drugs [3]. Long-term Mg deficiency is linked to increased risk of several aging-related diseases, from cardiovascular disease to osteoporosis [1,4]. Osteoporosis results from a negative balance between the bone-forming activities of osteoblasts and the resorptive activities of osteoclasts and is characterized by the loss of bone density that leads to fragility fractures. Interestingly, genetic hypomagnesemia with renal Mg wasting leads to low bone mass [5], and epidemiologic studies demonstrate that a correlation exists between Mg intake and bone mass and strength, thus associating inadequate dietary Mg to osteoporosis [5]. Although the effect of dietary Mg supplementation on bone mass in patients with osteoporosis has not been deeply investigated, available data indicate an increase in bone mineral density after Mg supplementation [6]. Several mechanisms are involved. Magnesium affects bone cell growth [7] as well as crystal formation [8]. In addition, individuals with Mg depletion have reduced 1,25(OH)2-vitamin D due to impaired parathyroid hormone (PTH) secretion and impaired activity of renal 1-alpha-hydroxylase, a Mg-dependent enzyme [9,10]. Interestingly, many osteoporotic postmenopausal women who are vitamin D deficient and have low PTH levels are Mg deficient [11], and Mg supplementation corrects these biochemical abnormalities. Moreover, hypomagnesemic diabetic children normalize their levels of 1,25(OH)2-vitamin D when supplemented with Mg [12].

In experimental Mg deficiency in rodents, decreased bone formation is observed, partly because of reduced osteoblastic activity [5]. Indeed, osteocalcin and alkaline phosphatase, markers of osteoblastic function, are decreased [13], and the number of osteoblasts detected by histomorphometry is reduced [14,15].

Osteoblasts are critical in creating and maintaining skeletal architecture. They are responsible for deposition of bone matrix and are also involved in the mineralization of osteoid. In addition, osteoblasts regulate the differentiation and activity of the bone-resorpting osteoclasts [16]. Inadequate osteoblast activity may result from a relative deficiency in proliferation or excessive apoptosis. As

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the cells responsible for bone formation, osteoblasts play a central role in the pathophysiology of osteoporosis [17].

Because (a) Mg deficiency decreases osteoblast proliferation in animal models and (b) Mg is mitogenic for osteoblasts in culture, we investigated the mechanisms involved in Mg-dependent regulation of SaOS-2 osteoblast-like cell proliferation.

2. Materials and methods

2.1. Cell culture, proliferation and transfection with small interfering RNA (siRNA) against inducible nitric oxide synthase (iNOS)

SaOS-2 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. These cells are commonly used as an osteoblastic model. Among many cell lines which exhibit osteoblastic features, we selected SaOS-2 cells because they exhibit the most mature osteoblastic phenotype among different osteoblast-like cell lines [18]. A Mg-free medium was purchased from Invitrogen (San Giuliano M.se, Italy) and utilized to vary the concentrations of Mg by the addition of $MgSO_4$ [19]. No significant difference was observed when we used $MgSO_4$ or $MgCl_2$ to add Mg to the culture media (not shown). One millimolar Mg is considered the physiological concentration of the cation. In all the experiments, the cells were seeded in growth medium; after 24 h, the medium was changed to expose the cells to various concentrations of Mg. Proliferation assays were performed on cells at low density (7500/cm^2) cultured in growth medium containing different concentrations of Mg. At various time points, the cells were trypsinized and stained with trypan blue solution (0.4%), and the viable cells were counted using a Burker chamber. In some experiments, cells were exposed to apocynin (10 μg/ml), Trolox (40 μM) or the selective iNOS inhibitor L-N^6-(iminoethyl)-lysine-HCl (L-NIL) (100 μM). To obtain a transient down-regulation of iNOS, we utilized the stealth siRNAs developed by Qiagen (Milano, Italy). The stealth siRNAs were transiently transfected into 3×10^5/cm^2 SaOS-2 using HyperFect Transfection Reagent (Qiagen). Protein extracts were made at 48 and 96 h to confirm gene down-regulation by western blot. Scrambled nonsilencing sequences (NS) were used as controls.

In other experiments, the cells were treated with the mitogen-activated protein kinase (MAPK) inhibitors ERK inhibitor III (10 μM), SB203580 and SP600125 (10 μM), or with the phosphatidylinositol-3 (PI3) kinase inhibitor LY294002 (10μM), all from Calbiochem (San Diego, CA, USA).

2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol reagent (Sigma-Aldrich, St Louis, MO, USA). One microgram of RNA was reverse transcribed and amplified by PCR. The sequences of the primers for iNOS are the following: 5′-GAGCTTCTACCTCAAGCTATC-3′ (forward); 5′-TGATGTTGCCATTGTTGGTGG-3′ (reverse) (Primm, Milano, Italy). The primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are the following: 5′-CCACCCATG GCAAATTCCATG GCA-3′ (forward) and 5′-TCTAGACGGCAG GTCAGGTCCACC-3′ (reverse) (Primm).

2.3. Reporter gene assay

Subconfluent SaOS-2 cells were transfected with a luciferase reporter plasmid (0.2 μg/cm^2) containing multiple copies of the NFkB consensus (pGL4.32[luc2P/NFkB-RE/Hygro]vector, Promega Italia, Milano, Italy) using Lipofectamine (Invitrogen). Cells were cotransfected with the pRL-TK plasmid encoding *Renilla* luciferase (5 ng/cm^2) as a control for differences in transfection efficiency. After 4 h, the cells were exposed for 24 h to either in 0.1, 1.0 or 5.0 mM Mg or lipopolysaccharide (LPS) 1 μg/ml as a positive control. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Italia), as described. Results are shown as the mean± standard deviation of three separate experiments in triplicate.

2.4. Immunoblot analysis

Cell extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose sheets at 200 mA for 16 h and probed with anti-platelet derived growth factor (PDGF)-BB, anti-interleukin (IL)-1, anti-IL-8, anti-IL-6 and anti-endothelial nitric oxide synthase (eNOS) antibodies (Santa Cruz Biotechnology-Tebu Bio, Magenta, Italy). Anti-Ser^{1177}-phosphorylated eNOS (eNOS/P-Ser^{1177}) and anti-iNOS were from Cell Signalling, Boston, MA, USA. Secondary antibodies were labeled with horseradish peroxidase (GE Healthcare, Milano, Italy). The SuperSignal chemiluminescence kit (Pierce, Rockford, IL, USA) was used to detect immunoreactive proteins. All the experiments were repeated at least three times with comparable results.

2.5. NOS activity

NOS activity was measured in the conditioned media by using the Griess method for nitrite quantification as described [20]. The concentrations of NO in the samples were determined using a calibration curve generated with standard $NaNO_2$ solutions. The experiments were performed in triplicate and repeated three to five times with similar results.

2.6. Statistical analysis

Statistical significance was determined using the Student's *t* test and set at *P* values less than .05. In the figures, *$P\leq.05$, **$P\leq.01$ and ***$P\leq.001$.

3. Results

3.1. Mg is required for SaOS-2 cell proliferation

We first cultured SaOS-2 cells in media containing concentrations of extracellular Mg ranging from 0.1 to 5.0 mM and counted them after 6 days. Five millimolars is a very high concentration which has been previously utilized in *in vitro* studies [21,22]; 0.1 mM Mg, a

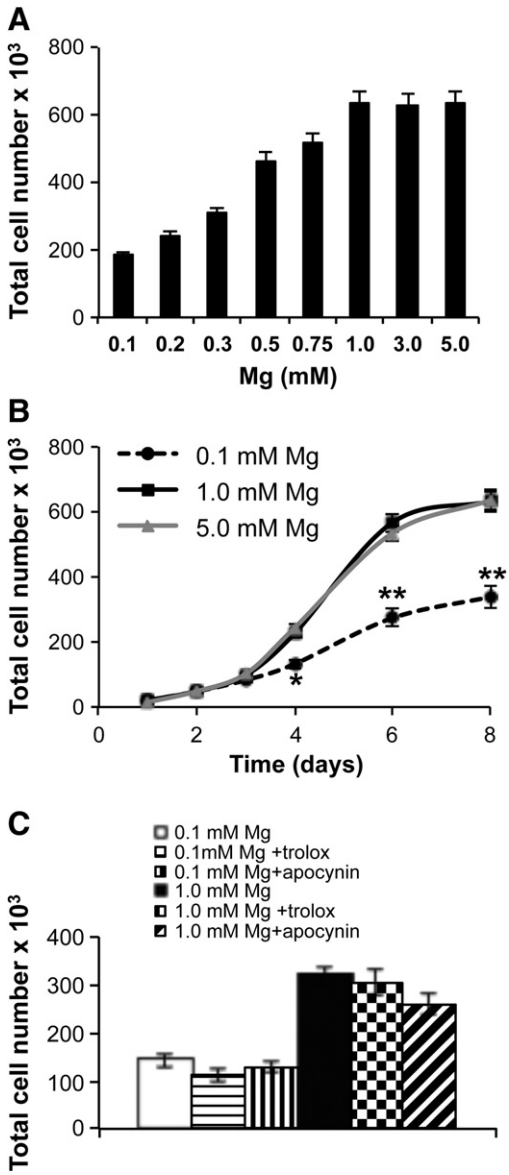

Fig. 1. Inhibition of SaOS-2 cell growth by low Mg concentration. (A) Viable SaOS-2 cells were counted after 6 days of culture in different concentrations of Mg. (B) Viable SaOS-2 cells cultured in 0.1, 1.0 and 5.0 mM Mg were counted every 24 h for 4 days and every 48 h for 4 additional days. Results are shown as the mean of three separate experiments± standard deviation (*$P\leq.05$; **$P\leq.01$). (C) SaOS-2 cells cultured in 0.1 or 1.0 mM Mg were treated with apocynin or Trolox, and viable cells were counted after 96 h. Results are shown as the mean of three separate experiments±standard deviation.

concentration which is detected in rodents after 8 days of experimental diet, has been widely used as an experimental model to study the effects of low Mg on cultured mammalian cells [21,23]. While high and physiologic extracellular Mg yielded similar results, low extracellular Mg impaired SaOS-2 cell growth in a dose-dependent fashion (Fig. 1A). On the basis of these results, to better highlight differences in cell behavior, we compared the growth of SaOS-2 cells cultured for different times in 0.1, 1.0, and 5.0 mM Mg. SaOS-2 cells in 0.1 mM Mg were significantly growth-inhibited after 4 days and up to day 8 (Fig. 1B), while no differences were observed between cells in 1.0 and 5.0 mM Mg.

Because 96 h is the first time point in which the inhibition of proliferation in 0.1 mM Mg is significant, all the subsequent experiments aimed at investigating the mechanisms mediating growth inhibition by low Mg were performed after 96 h of culture in 0.1 mM Mg. In various cell types, Mg deficiency has been associated with oxidative stress [19,24]. We therefore evaluated whether antioxidants might prevent the inhibition of SaOS-2 cell growth by low Mg. Fig. 1C shows that apocynin, a NADPH oxidase inhibitor, and Trolox, a water-soluble analog of α-tocopherol which protects against lipoperoxidation, did not rescue SaOS-2 cell growth after 96 h in 0.1 mM Mg. These results suggest that oxidative stress is not involved in mediating low Mg-dependent inhibition of cell growth.

3.2. Mg does not promote an inflammatory phenotype in SaOS-2 cells

Because Mg is implicated in the regulation of the inflammatory response *in vivo* and *in vitro* [19,25] and cytokines regulate cell growth, we evaluated whether different concentrations of extracellular Mg impact on the activation of NFkB, the master regulator of inflammation, and consequently on the production of cytokines. We first investigated the functional induction of NFkB by reporter gene assays using a vector expressing luciferase under the control of multiple copies of the NFkB responsive consensus [19]. The construct was transiently transfected into SaOS-2 cells, and the activity of luciferase was monitored as described in the methods. No modulation of luciferase activity was observed in cells exposed to 0.1, 1.0 and 5.0 mM Mg (Fig. 2A). LPS (1 μg/ml) was used as positive control of NFkB activation. Accordingly, by protein array tailored for proteins involved in inflammation, we did not detect any significant alteration of the cytokine network (not shown). Western blot analysis confirmed that Mg did not affect the total amounts of IL-1, IL-6, IL-8 and PDGF-BB, all targets of NFkB [19] (Fig. 2B).

3.3. Mg modulates the release of nitric oxide

Because of the role of NO in bone homeostasis [26], we evaluated whether Mg affected NOS activity. After 96 h of culture in various concentrations of Mg, we found that NOS activity was higher in SaOS-2 cells exposed to 0.1 mM Mg than in cells cultured in 1.0 or 5.0 mM Mg (Fig. 3A). In cells exposed to 0.1 mM Mg, the amounts of eNOS and its phosphorylated form on Ser^{1177}, which has higher enzymatic activity [27], remained unvaried as evaluated by western blot (Fig. 3B). Interestingly, iNOS is up-regulated in cells cultured in 0.1 mM Mg vs. their controls (Fig. 3B), and down-regulating iNOS with siRNA, as demonstrated by western blot (Fig. 3C), abolished low Mg-induced NO release (Fig. 3D).

In agreement with the evidence that iNOS is mainly regulated at the transcriptional level, by semiquantitative RT-PCR, we found an up-regulation of iNOS transcript in cells in 0.1 mM vs. cells in 1.0 or 5.0 mm Mg (Fig. 4A). Different signaling molecules are involved in iNOS induction [28]. We investigated the involvement of MAPK and phosphatidylinositol-3 (PI3)-kinase signaling in modulating iNOS levels by low Mg. We therefore exposed the cells to MAPK inhibitors of ERK (ERK inhibitor III), p38 (SB203580), JNK (SP600125) and PI3-kinase (LY294002) activities. While the inhibitors of PI3-kinase and p38 exerted no effects, the inhibitor of JNK totally abrogated iNOS induction in low Mg. Even though ERK inhibitor markedly down-regulated iNOS in cells cultured in both 1.0 and 0.1 mM Mg, the amounts of iNOS remained higher in SaOS-2 cells in low Mg than in the controls (Fig. 4B).

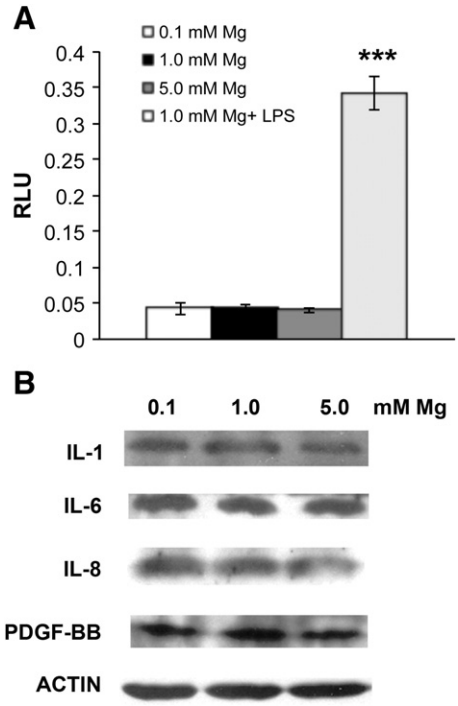

Fig. 2. Effect of different concentrations of Mg on the induction of NF-KB activity. (A) Luciferase assay was performed on SaOS-2 cultured in 0.1, 1.0 and 5.0 mM Mg as described in the methods. Treatment with LPS 1 μg/ml was used as a positive control. Luciferase activity was detected and analyzed by fluorimetry. Histograms represent the mean of three separate experiments performed in triplicate±standard deviation (***$P \leq .001$). (B) Protein extracts (80 μg) prepared from SaOS-2 cultured for 72 h in 0.1, 1.0 and 5.0 mM Mg were separated on SDS-PAGE and processed for western blot with specific antibodies against IL-1, IL-6, IL-8 and PDGF-BB. The filter was then probed for actin to show that comparable amounts of protein were loaded per lane.

3.4. Inhibition of iNOS activity rescues SaOS-2 cell proliferation

To study whether an increased activity of iNOS was responsible for SaOS-2 cell growth retardation by low Mg, the cells were incubated in 0.1 mM Mg containing medium in the presence or in the absence of the iNOS inhibitor L-NIL (100 μM) and counted after different times. Fig. 5A shows that SaOS-2 cells cultured in 0.1 mM Mg in the presence of L-NIL proliferated as fast as the controls, thus indicating that NO is responsible for SaOS-2 cell growth inhibition by low Mg. Similar results were obtained also with the NOS inhibitor L-NG-Nitroarginine methyl ester (L-NAME) but not with its inactive enantiomer D-NG-Nitroarginine methyl ester (D-NAME) (data not shown). We also silenced iNOS by transiently transfecting the cells with siRNA anti-iNOS. Our controls were cells transfected with a random siRNA (NS). Fig. 5B shows that iNOS silencing rescues SaOS-2 cell proliferation in 0.1 mM Mg to levels comparable to the controls.

4. Discussion

Since it is an inevitable consequence of aging, osteoporosis is a major health concern considering that the aging population will double over the next decade. Osteoporosis results from an unbalance

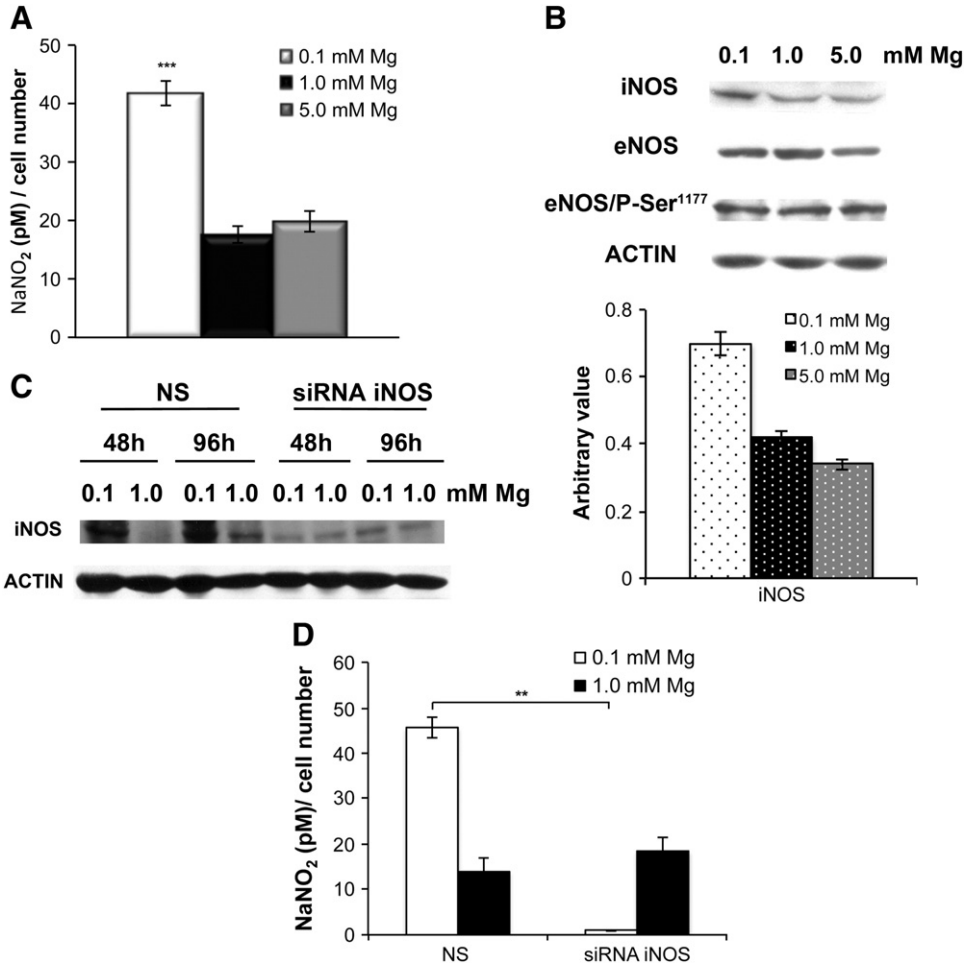

Fig. 3. Effect of Mg on the release of nitric oxide. (A) Cells were cultured in 0.1, 1.0 and 5.0 mM Mg. NO was measured according to the Griess method for nitrite quantification after 96 h of culture. Results are expressed as the mean±standard deviation of three different experiments (***$P \leq .001$). (B) SaOS-2 cells exposed to different concentrations of Mg for 72 h were lysed, and 80 μg of protein extracts was loaded on SDS-PAGE. Western blots using specific antibodies against iNOS, eNOS and eNOS-P-Ser^{1177} were performed. Actin shows that equal amounts of protein were loaded per lane. The figure shows a representative blot. The histogram shows the quantitative evaluation of iNOS/actin ratio by densitometry. Results are expressed as the mean±standard deviation of three separate experiments. (C) SaOS-2 cells in 0.1 or 1.0 mM Mg were transiently transfected by siRNA against iNOS or NS. Western blot with anti-iNOS antibodies was performed on 100 μg of cell extracts 48 and 96 h after transfection. Actin is used as a control of loading. (D) Cells were cultured in 0.1 and 1.0 mM Mg and transiently transfected with siRNA against iNOS or with an NS. NO was measured as described. Results are expressed as the mean±standard deviation of three different experiments (**$P \leq .01$).

between bone deposition and resorption. Osteoblasts ensure bone formation and mineralization through secretion of bone matrix components (type I collagen and noncollagenous proteins) and also play a central role in the regulation of bone resorption by synthesizing factors essential for the differentiation of osteoclasts [16]. Therefore, adequate osteoblast proliferation, differentiation, secretory function or rate of apoptosis is essential for both adequate formation and resorption processes and thereby for the maintenance of bone remodeling equilibrium. It has been established that, with aging, the process of bone formation is affected by the reduction of osteoblast proliferation and life span [29].

Osteoporosis results from complex interactions among local and systemic regulators of bone cell function [17]. Among others, Mg has a role in bone physiology, and its deficiency associates with osteoporosis [5,30]. Because Mg is required for cell proliferation [31], it was not surprising that low Mg inhibited SaOS-2 cell growth. Interestingly, the inhibitory effect was significant after 96 h of exposure to Mg-deficient medium, suggesting that these cells need to adapt to low Mg concentration before growth retardation becomes evident. On the contrary, human endothelial cells are rapidly growth inhibited after exposure to Mg-deficient medium [32]. We argue that different cell types might utilize different pathways to adapt to Mg deficiency.

We then investigated how low Mg brakes SaOS-2 cell growth. Because Mg deficiency has been linked to oxidative stress, we evaluated whether antioxidants could prevent growth inhibition by low Mg. We found that two different antioxidants which act through different mechanisms of action have no effect in preventing SaOS-2 cell growth inhibition by low Mg. Since low Mg promotes the acquisition of a inflammatory phenotype *in vitro* and *in vivo* [26,33], we next evaluated the cytokine profile of SaOS-2 cells cultured in low Mg. Indeed, certain proinflammatory cytokines play critical roles both in the normal bone remodeling process and in the pathogenesis of perimenopausal and late-life osteoporosis [34]. For example, IL-6 promotes osteoclast differentiation and activation [34], and IL-1 is another potent stimulator of bone resorption [35] that has been linked to the accelerated bone loss in idiopathic and postmenopausal osteoporosis [36]. We here show that culture of SaOS-2 cells in Mg-deficient medium does not activate NFkB and, accordingly, does not up-regulate various cytokines, including IL-1 and IL-6, and growth factors, among which is PDGF-BB, which induces human osteoblast cell proliferation and migration and is involved in bone remodeling. Interestingly, in endothelial cells, we have shown that low Mg induces IL-1, which is responsible for the inhibition of cell proliferation and the up-regulation of vascular cell adhesion molecule-1 [37]. Recently,

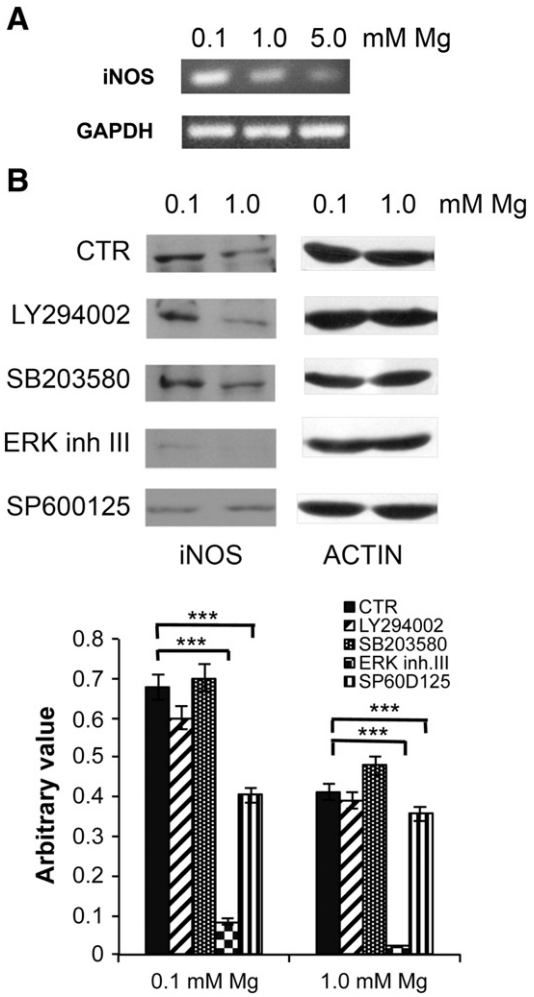

Fig. 4. Effects of MAPK and PI3-kinase inhibitors on iNOS levels. (A) RT-PCR was performed on SaOS-2 cells cultured in 0.1, 1.0 or 5.0 mM Mg for 24 h. GAPDH is used as a control of loading. (B) SaOS-2 cells in 0.1 or 1.0 mM Mg were exposed to ERK inhibitor III, p38 inhibitor SB203580, JNK1/2 inhibitor SP600125 and PI3-kinase inhibitor LY294002 for 48 h. Western blot was performed with antibodies against iNOS. Actin was used a marker of loading. The histogram shows the quantitative evaluation of iNOS/actin ratio by densitometry. Results are expressed as the mean±standard deviation of three separate experiments (***$P \leq .001$).

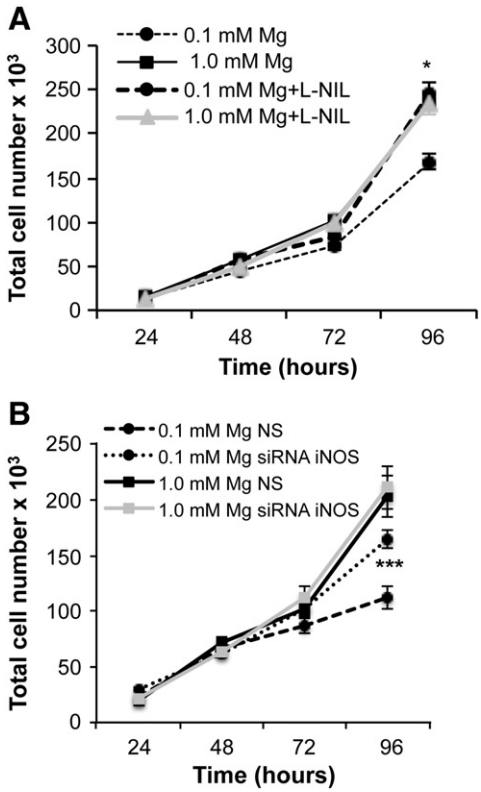

Fig. 5. Nitric oxide is responsible for low Mg inhibition of cell growth. (A) Cells cultured in 0.1 or 1.0 mM Mg were treated every 24 h with L-NIL, a specific inhibitor of iNOS, and counted at different time points. Data are expressed as the mean±standard deviation of four independent experiments (*$P \leq .05$). (B) iNOS was transiently silenced in cells cultured in 0.1 or 1.0 mM Mg by siRNA against iNOS or NS. The cells were counted every 24 h for 4 days. Data are expressed as the mean±standard deviation of three independent experiments (0.1 mM Mg NS vs. 0.1 mM Mg siRNA iNOS, ***$P \leq .001$).

we have demonstrated the acquisition of an inflammatory phenotype in endothelial cells because of the activation of NF-kB via free radicals [19]. We therefore hypothesize that *in vivo* endothelial cells of bone vasculature might create a proinflammatory microenvironment by releasing inflammatory mediators that could ultimately affect bone structure in Mg deficiency. Also, osteoclasts play a role in promoting inflammation within the bone environment. Indeed, when compared to controls, osteoclasts of Mg-deficient rats produce higher amounts of tumor necrosis factor (TNF)-α and substance P, the latter being involved in increasing the release of IL-1 and IL-6 by bone marrow cells [30]. Another source of inflammatory cytokines in *in vivo* models of Mg depletion could be peripheral blood monocytes which produce high amounts of IL-1, IL-6 and/or TNF-α [25]. Altogether, these changes in the cytokine network offer an explanation for the uncoupling of bone formation and bone resorption observed in Mg-deficient animals.

NO, which is synthesized by osteoblasts through the activity of two isoforms of NOS, also contributes to the pathogenesis of osteoporosis [38]. eNOS is widely expressed in bone on a constitutive basis, whereas iNOS is mainly expressed in response to inflammatory stimuli. eNOS seems to play a key role in regulating osteoblast activity and bone formation since eNOS knockout mice have osteoporosis due to defective bone formation. Also, iNOS null mice show imbalances in bone osteogenesis and abnormalities in bone healing [38]. Moreover, iNOS pathway is essential for IL-1-induced bone resorption and also mediates the negative effects of estrogen depletion on bones [39]. While the constitutive production of NO at low concentrations promotes the proliferation of osteoblast-like cells and modulates osteoblast function, a moderate induction of NO is sufficient to potentiate bone resorption [26]. In particular, the activation of the iNOS pathway inhibits osteoblast function *in vitro* and stimulates osteoblast apoptosis [40]. It is noteworthy that we found that SaOS-2 cells cultured in low Mg up-regulate iNOS and, because of this, produce higher amounts of NO. Importantly, both pharmacological and genetic inhibition of iNOS restored the normal proliferation rate of the cells cultured in low extracellular Mg. Signaling pathways resulting in the induction of iNOS expression vary in different cell types or species [28]. In general, modulation of iNOS expression is the most important component of iNOS regulation, through the activation of several signaling pathways, among which are NFkB, MAPK and the Janus kinase/signal transducer and activator of transcription. In SaOS-2 cells cultured 0.1 mM Mg, we rule out a role of NFkB, which is not activated and, therefore, involved in iNOS up-regulation. We found that JNK is necessary for iNOS induction by low Mg in SaOS-2 cells. Indeed, the inhibitor of JNK1/2 SP600125 prevented iNOS up-regulation in cells in 0.1 mM Mg. That JNK has a role in regulating iNOS has been previously shown, since iNOS protein expression was abolished by the JNK inhibitor in microvascular endothelial cells exposed to LPS [41] and in hyperglycemia-induced iNOS expression in diabetic mice embryos [42].

It is noteworthy that JNK signaling has been implicated in a number of important physiological processes, from embryonic morphogenesis to cell survival and apoptosis [43], and has also been linked to human diseases, including tumor development, cardiac hypertrophy, ischemia/reperfusion injury, diabetes, hyperglycemia-induced apoptosis and several neurodegenerative disorders [43,44]. It is known that the addition of Mg inhibited oxidized low-density lipoprotein-induced JNK activation in human endothelial cells [45], thereby indicating that Mg contributes to the regulation of JNK signaling. However, in our experimental model, more experiments are necessary to clarify the link between Mg and JNK regulation.

Briefly, our results indicate that low Mg impairs osteoblast-like SaOS-2 cell proliferation through the up-regulation of iNOS and consequent induction of NO release. We therefore suggest that a nutritional protocol aimed at maintaining proper concentrations of extracellular Mg might contribute to ensure normal growth and function of osteoblasts and, therefore, to prevent demineralizing bone diseases.

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