Low-magnesium induces senescent features in cultured human endothelial cells

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Abstract. Low magnesium (Mg) affects endothelial function, thus playing a role in cardiovascular diseases, including atherosclerosis. We here show that Mg deficiency acts through the induction of the pro-inflammatory cytokine interleukin (IL)-1α in cultured human endothelial cells. Indeed, the inhibition of IL-1α prevents low Mg-induced adhesion of monocytoid cells to the endothelium as well as the upregulation of the cdk inhibitor p21. We also demonstrate that Mg deficiency induces several features typically associated with endothelial senescence. While low Mg can affect gene expression at the transcriptional level, it also modulates the activity of the proteasome. Since endothelial senescence contributes to atherogenesis, our findings indicating that low Mg promotes senescence may shed some light on the molecular mechanisms linking Mg deficiency to cardiovascular diseases.

Key words: endothelial cell, magnesium, interleukin 1, senescence

Magnesium (Mg) plays an essential role in fundamental cellular reactions [1]. Several in vitro studies have provided evidence for the interactions of Mg with phospholipids, proteins and nucleic acids. In particular, Mg influences the catalytic activity of various enzymes and this is well exemplified by the ability of Mg to promote trans-phosphorylation reactions through the formation of ATP-Mg complexes which anchor substrates to the active sites of enzymes [1].

On these bases, it is not surprising that perturbations of plasma concentrations of Mg have been linked to various clinical settings, including cardiovascular diseases, diabetes, hypertension and arrhythmias [2]. Mg insufficiency, which is frequent in western countries, has been suggested as a contributory factor to the development of coronary artery disease [3]. In patients with coronary artery disease, oral Mg therapy has been associated with a significant improvement of endothelial function [4] and with a decrease of plasma concentrations of triglycerides, VLDL and apolipoprotein B [5]. In addition, oral Mg has favorable effects on left ventricular function during rest and exercise in stable coronary artery disease patients [6].

During experimental Mg deficiency hyperlipemia, inflammation and early atherosclerotic lesions have been observed [7]. Interestingly, Mg fortification of drinking water succeeded in the inhibition of atherosclerosis in APO-E-deficient mice fed with a high cholesterol diet [8]. Similar results were obtained in LDL receptor-deficient mice on a high fat diet, which develop severe hypercholesterolemia and advanced atherosclerosis. These detrimental events are retarded after Mg fortification of the drinking water which, however, does not affect the lipid profile [9]. This result is puzzling, since it suggests that Mg acts on targets other than hyperlipemia.

Vascular diseases, including atherosclerosis, recognize the endothelial cell as an important player, because an endothelial dysfunction is the common link between risk factors and atherosclerotic burden. Indeed, endothelial cells in low Mg increase LDL
transport across the endothelial monolayer, enhance the oxidation of LDL, stimulates the secretion of Platelet Derived Growth Factor (PDGF) and upregulate adhesion molecules and plasminogen activator inhibitor-1 [10, 11], all pivotal steps in the onset of the atherosclerotic lesion. In agreement with the in vivo evidence that low Mg promotes inflammation, endothelial cells grown in low Mg upregulate interleukin (IL)-1α [11]. IL-1α induces endothelial activation and promotes endothelial senescence [12]. We here demonstrate that the overexpression of IL-1α in cells in low Mg is critical in mediating some of low Mg effects on endothelial cells. In addition, we show that low Mg promotes a senescent phenotype in human endothelial cells.

Materials and methods

Cell culture and proliferation assay
Human umbilical vein endothelial cells (HUVEC-C from ATCC, Manassas, VA, USA) were cultured in M199 containing 10% fetal calf serum (FCS), Endothelial Cell Growth Supplement (150 μg/mL) and heparin (5 U/mL) on 2% gelatin coated dishes. A Mg free medium was purchased from Invitrogen (Milano, Italy) and used to vary the concentrations of Mg by the addition of MgSO4 [11]. In all the experiments the cells were seeded in growth medium; after 12 h, the medium was changed to expose the cells to various concentrations of Mg. For proliferation assays HUVEC (7500/cm2) cultured in different concentrations of Mg for 96 h were trypsinized, stained with trypan blue solution (0.4%) and the viable cells were counted using a Bürker chamber. In some experiments, HUVEC were incubated in the presence of an IL-1α antisense oligomer (50 μM) [11] or, as a control, with the corresponding sense oligomer. All the experiments have been repeated at least three times in triplicate. The results are shown as the mean ± SD.

Adhesion assay
Confluent HUVEC were cultured in 24-well plates with 0.1 or 1.0 mM Mg for 96 h in the presence of a daily added oligomer against IL-1α or its control. The wells were rinsed three times and 2x10⁵ U937 cells were added to each well. After 3 h, the non adherent U937 cells were rinsed off and the wells were fixed with 1% glutaraldehyde. The number of attached U937 cells was counted in twenty microscopic fields defined by an eyepiece. The experiments were performed in triplicate at least three times [11]. The results are shown as the mean ± SD.

Western blot
HUVEC were lysed in 10 mM Tris-HCl (pH 7.4) containing 3 mM MgCl₂, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protein inhibitors, separated on 12% SDS-PAGE and transferred to nitrocellulose sheets. Western blot analysis was performed using polyclonal antibodies against human p21, IL-1α and GAPDH (Tebu-bio-Santa Cruz, Santa Cruz, CA, 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.

Determination of proteasome activity
HUVEC were lysed in 20 mM Tris-HCl containing 10% glycerol, 5 mM ATP and 0.2% NP-40. After centrifugation, the proteasome activity was determined as described [13]. The samples were quantified using a luminescence spectrometer at 380 nm (excitation) and 460 nm (emission). The proteasome activity was calculated on the standard activity curve of the 20S Proteasome Positive Control (Chemicon, Hampshire, United Kingdom) and expressed in pM. The results are the mean ± SD of two separate experiments in triplicate.

Results

Low Mg alters endothelial behavior
1.0 mM Mg is considered the physiological concentration of the cation. In humans, the lowest level of Mg in critically ill subjects can reach 0.5 mM [14] and the mean concentration is 0.772 +/- 0.58 (SD) mmol/L [10, 15, 16]. In rodents, after 8 days of experimental diet, a decrease in plasma Mg concentration to 0.14 mM can be detected [17]. Thus, we used concentrations ranging from 0.1 to 1.0 mM Mg.

Figure 1 shows the morphologic change of HUVEC when cultured in 0.1 compared to 1.0 mM of extracellular Mg for 5 days. In low Mg, the cells were vacuolated and show an increase in size, both features of senescence. Since senescence associates with growth inhibition, it is noteworthy that low Mg inhibited endothelial proliferation in a dose-dependent manner (figure 2A). Figure 2B shows that low Mg upregulated p21 (WAF1), an inhibitor of cyclin-dependent kinases (cdk).
Low Mg enhances IL-1α synthesis

IL-1α is a potent inhibitor of endothelial cell growth and is a marker of endothelial senescence [12, 18]. We therefore evaluated the expression of IL-1α in HUVEC grown in 0.1 vs 1.0 mM Mg for 24 and 72 h by western blot. We found that cells cultured in low Mg produce higher amounts of the cytokine than controls in a time dependent fashion (figure 3).

Low Mg effects are mediated by IL-1α

Since an antisense oligomer against IL-1α prevented the growth inhibition of HUVEC grown in Mg-deficient medium [11], we evaluated the effect of the oligomer on the expression of p21. As demonstrated in figure 4A, the inhibition of IL-1α translation inhibited the accumulation of p21 in HUVEC cultured in 0.1 mM Mg. We have also demonstrated that low Mg enhanced endothelial/monocyte interactions. Since IL-1α induces the expression of adhesion molecules, we cultured the cells in low Mg for 96 h in the presence of the anti-IL-1α oligomer and evaluated the interaction with the monocytoid U937 cells. We observed a decrease in the number of U937 cells bound to the endothelial monolayer when IL-1α translation was inhibited (figure 4B).

Low Mg enhances proteasome activity

We measured proteasome activity on crude extracts of HUVEC cultured in 0.1 vs 1.0 mM Mg for 96 h. Table 1 shows a higher activity in cells in 0.1 mM Mg than in 1.0 mM.

Discussion

Since endothelial dysfunction is a key event in atherogenesis, it is noteworthy that low Mg, which induces endothelial dysfunction in vitro, contributes to the development of atherosclerosis in vivo [10, 11]. Interestingly, endothelial cells with senescence associated phenotypes are present in human atherosclerotic lesions and endothelial senescence contributes to atherogenesis [19]. We here provide evidence that low Mg promotes the acquisition of some features which are typically associated with endothelial senescence. We show that HUVEC in low Mg resemble senescent cells because they become large, elongated and vacuolated. In addition, they are growth inhibited and upregulate p21, a cdk inhibitor which determines the accumulation of cells in the G1 phase of the cell cycle. p21 has been shown to play a role in senescence, since its forced expression induces premature senescence associated with cell dysfunction in cultured endothelial cells [20].

The overexpression of IL-1α specifically characterizes endothelial senescence [12]. Interestingly, low Mg induces IL-1α in endothelial cells. This is in agreement with the findings that Mg deficiency promotes a pro-inflammatory response in vivo [7] which is known to be crucial in atherogenesis. IL-1α causes multiple responses in vascular endothelial cells including inhibition of cell proliferation and induction of adhesion molecules which bind leukocytes. We therefore used an antisense oligonucleotide against IL-1α to block its translation. HUVEC negative for IL-1α expression and cultured in low Mg

Figure 1. Low Mg medium alters endothelial morphology in vitro. Microphotographs of HUVEC cultured in 0.1 or 1.0 mM Mg were taken under phase-contrast microscopy.
recuperate their normal growth rate [11] and down-regulate p21. In addition, the antisense oligomer markedly reduces U937/endothelial interactions. These results indicate that IL-1α mediates some of the effects of low Mg in endothelial cells.

Endothelial cells upon exposure to low Mg undergo profound alterations of function that involve the modulation of gene expression and protein synthesis/degradation. We have previously shown that low Mg alters gene expression at the transcriptional level [11]. Here we show that low Mg induces proteasome activity in endothelial cells. Since the proteasome activator subunits Red Blood Cell Regulators (REG) require calcium for their activity [21] and Mg is the physiologic calcium antagonist, we hypothesize that culture in low Mg could increase intracellular calcium, with consequent potentiation of REG activity. Since the proteasome activity is higher in senescent than in young endothelial cells [13], these results underscore that low Mg induces the acquisition of a senescent phenotype. Since growth inhibition usually correlates with a reduction of protein synthesis, we propose that the increased proteolytic activity in endothelial cells in low Mg may contribute to the metabolic homeostasis of the cells. Alternatively, it is possible that more unfolded or damaged proteins accumulate in cells grown in low Mg, rendering the activation of the

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<th>Mg concentration (mM)</th>
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<tr>
<td>0.1</td>
<td>16.8 ± 1.2</td>
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<tr>
<td>1.0</td>
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Proteasome activity was determined using the 20S proteasome activity assay kit (Chemicon, Hampshire, United Kingdom) according to the manufacturer’s instructions. The proteasome activity was calculated on the standard activity curve of the 20S proteasome positive control. The results are the mean ± standard deviation of two separate experiments in triplicate.
proteasome a crucial event for cell survival. Further studies are required to understand the mechanisms underlying this activation of the proteasome in low Mg.

Conclusion

We conclude that Mg deficiency has a complex role in atherogenesis. Apart from inducing hyperlipemia and inflammation which are pivotal in this process, low Mg affects endothelial cells by modulating their functions and promoting senescence, mainly through the action of IL-1α.

References


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**Figure 4.** IL-1α mediates some of low Mg effects on endothelial cells. HUVEC were cultured for 96 h in 0.1 mM Mg containing medium in the presence of an antisense oligomer against IL-1α or its control. A) Lysates were utilized for western blot with an anti-p21 antibodies as described. An anti-GAPDH antibody was used to show that comparable amounts of proteins were loaded per lane. B) Adhesion assay was performed as described in the methods. The experiment was performed in triplicate and results are shown as the mean ± SD.


