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**MAGNESIUM DEFICIENCY PROMOTES A PROATHEROGENIC PHENOTYPE IN  
CULTURED HUMAN ENDOTHELIAL CELLS VIA ACTIVATION OF NF $\kappa$ B.**

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**ABSTRACT**

Phenotypic modulation of endothelium to a dysfunctional state contributes to the pathogenesis of atherosclerosis, partly through the activation of the transcription factor NFκB. Several data indicate that magnesium deficiency caused by prolonged insufficient intake and/or defects in its homeostasis may be a missing link between diverse cardiovascular risk factors and atherosclerosis. Here we report that endothelial cells cultured in low magnesium rapidly activate NFκB, an event which is prevented by exposure to the anti-oxidant trolox. It is well known that NFκB activation correlates with marked alterations of the cytokine network. In the present study, we show that exposure of endothelial cells to low magnesium increases the secretion of RANTES, interleukin 8 and platelet derived growth factor BB, all important players in atherogenesis. Moreover, we describe the increased secretion of matrix metalloprotease-2 and -9 and of their inhibitor TIMP-2. Interestingly, by zymography we show that metalloprotease activity predominated over the inhibitory effect of TIMP-2.

These results indicate that low magnesium promotes endothelial dysfunction by inducing pro-inflammatory and pro-atherogenic events.

Keywords: endothelial cells, magnesium, NFκB, cytokines, MMP

Abbreviations: Magnesium Mg, matrix metalloprotease MMP, inhibitor of metalloprotease TIMP, interleukin IL, granulocyte macrophage-colony stimulating factor GM-CSF, platelet derived growth factor PDGF, tumor necrosis factor TNF, coronary artery disease CAD, Vascular Cell Adhesion Molecule VCAM, plasminogen activator inhibitor PAI, human umbilical vein endothelial cells HUVEC, lipopolysaccharide LPS, standard deviation SD

## 1. INTRODUCTION

Vascular endothelium, which comprises a monolayer of more than ten trillion endothelial cells, represents a dynamic interface between the blood and the surrounding tissues [1]. Apart from forming a selective semi-permeable barrier, the endothelial cells actively and reactively control vascular tone and hemostasis as well as the ingress and egress of leukocytes. Consequently, it is not surprising that endothelial dysfunction is the common denominator that links cardiovascular risk factors and some diseases (diabetes, hyperlipemia, hypertension) to atherosclerotic burden [2]. Indeed, endothelial dysfunction contributes to the initiation and progression of atherosclerotic disease and has been suggested to be an independent vascular risk factor [3].

Also magnesium (Mg) deficiency has been reported to be involved in the pathogenesis of cardiovascular diseases in general and of atherosclerosis in particular [4]. Indeed, Mg fortification of drinking water succeeded in the inhibition of atherogenesis in APO-E-deficient mice fed with a high cholesterol diet [5] and oral Mg therapy has been associated with significant improvement of endothelial function in patients with coronary artery disease (CAD) [6]. In addition, the Atherosclerosis Risk in Communities Study concluded that the highest risk for CAD occurred in subjects with the lowest serum Mg, even after controlling for the traditional CAD risk factors [7]. Evidence suggests that the occidental diet is relatively deficient in Mg because of preference for calorie-rich, micronutrient-poor foods, low Mg content in water and soil, and processing of many food items [8]. Moreover, it should be recalled that Mg deficiency is a significant clinical complication arising in patients with diabetes mellitus, in nephropathics, in individuals treated with some classes of diuretics or anticancer drugs, as well as in alcoholists. Hypomagnesemia is also common in hospitalized patients, in particular in the elderly [8].

In cultured endothelial cells, low Mg increases the adhesion of monocytes to cultured endothelial cells via the upregulation of Vascular Cell Adhesion Molecule (VCAM), induces plasminogen activator inhibitor (PAI) 1, augments the levels of the pro-inflammatory cytokine interleukin (IL)-1 alpha, impairs endothelial proliferation and promotes cellular senescence [9-10]. Recently, endothelial function has been shown significantly impaired in a model of inherited hypomagnesemia in mice (MgL mice) [11]. In agreement with the results obtained in cultured endothelial cells, MgL aortas had higher expression of VCAM and PAI-1 than their controls [11]. In addition, in Mg deficient rodents an inflammatory response has been described [12]. All these results point to the fact that low Mg promotes the acquisition of an inflammatory phenotype in the endothelium.

The family of transcription factors named NFkB controls the global inflammatory response and

regulates the proatherogenic program in endothelial cells [13]. In resting cells, NFkB subunits are held in the cytosol because of their association with inhibitory proteins denominated IkB [13]. Upon cytokine signalling or environmental stress, NFkB activation is initiated [14]. The canonical pathway of NFkB activation involves the rapid degradation of IkB by the proteasome, the release of NFkB subunits, their translocation to the nucleus and binding to the cognate DNA motifs in target genes.

Because i) endothelial dysfunction, early event in atherogenesis, is driven by NFkB [14], and ii) Mg deficiency affects endothelial behaviour and contributes to atherosclerosis [4, 9], we investigated NFkB in human umbilical vein endothelial cells (HUVEC) cultured in low Mg. We show that Mg deficiency activates NFkB and this event associates with the overexpression of the chemokines IL-8 and RANTES, of platelet derived growth factor (PDGF)-BB, Tissue Inhibitor of Matrix Metalloproteases (TIMP)-2 and metalloproteases (MMP) 2 and 9, all implicated in inflammation and atherogenesis.

## 2. MATERIALS AND METHODS

**2.1. Cell culture and reporter gene assay.** Human umbilical vein endothelial cells (HUVEC) were cultured in M199 containing 10% fetal bovine serum, 1 mM glutamine, 1 mM penicillin and streptomycin, Endothelial Cell Growth Factor (ECGF, 150 µg/ml), 1 mM sodium pyruvate and heparin (5 units/ml) on 2% gelatin-coated dishes [9]. A magnesium free medium was purchased by Invitrogen (San Giuliano M.se, Italy) and utilized to vary the concentrations of magnesium by the addition of MgSO<sub>4</sub> [9]. On the basis of previous reports [9], we cultured HUVEC in medium containing 0.1 or 1.0 mM. In all the experiments the cells were seeded in growth medium; after 24 h, the medium was changed to expose the cells to either 0.1 or 1.0 mM Mg. In some experiments HUVEC were exposed to lipopolysaccharide (LPS)(1 µg/ml) or trolox (40 µM) (all from Sigma Aldrich, St Louis, MO). Dose response experiments with trolox indicated that in our cells 40 µM is the lowest concentration reproducibly yielding an effect. HUVEC were pretreated with trolox for 1 h and then maintained in the presence of trolox for the duration of the experiment.

For reporter gene assays, subconfluent HUVEC were transfected with a luciferase reporter plasmid (0.2 µg/cm<sup>2</sup>) containing multiple copies of the NFκB consensus (pGL4.32[luc2P/NFκB-RE/Hygro]vector, Promega Italia, Milano, Italy) using Arrest-in (Invitrogen). Cells were cotransfected with the phRL-TK plasmid encoding *Renilla* luciferase (5 ng/cm<sup>2</sup>), as a control for differences in transfection efficiency. After 4 hours, the cells were exposed overnight to either in 0.1 or 1.0 mM Mg. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Italia, Milano, Italy), according to the manufacturer's instruction Firefly luciferase activity was normalized to *Renilla* luciferase activity. Results are shown as the mean ± standard deviation (SD) of three separate experiments in triplicate.

**2.2. Electrophoretic Mobility Shift Assays (EMSA).** Nuclear extracts were prepared and protein concentration was determined with Bradford reagent (Sigma Aldrich). Single strand oligonucleotide with consensus binding-sites of NFκB (5'-GATCCTCAACAGAGGGGACTTTCCGAGGCCA-3') was labelled with T4 polynucleotide kinase (GE Healthcare, Milano, Italy) by using <sup>32</sup>P-ATP, annealed to the complementary strand (5'-TGGCCTCGGAAAGTCCCCTCTGTTGAGGATCC-3'), and then purified by polyacrylamide gel electrophoresis. 5 µg of nuclear extract were incubated for 30 minutes at room temperature with 20 Kcpm of <sup>32</sup>P-labelled probe in binding buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 5% glycerol, 0.5 mM DTT). All binding reactions contained 0.5 µg poly (dI-dC) as a nonspecific competitor. For competition experiments, 50-fold excess of unlabelled specific or unrelated double-stranded sequences was added to the binding

mixtures. For supershift experiments, nuclear extracts were incubated with antibodies (1  $\mu$ g) against p65 and p50 (Santa Cruz Biotechnology-Tebu Bio, Magenta, Italy) for 60 min on ice followed by 25 min of incubation with oligonucleotide.

Protein-DNA complexes were resolved on 5% acrylamide gels. Gels were dried and radiolabelled bands detected by autoradiography. The experiments were repeated three times with similar results.

*2.3. Protein Array.* HUVEC were cultured in medium containing 0.1 or 1.0 mM Mg for 2 days. 5 ml of conditioned media were centrifuged and utilized to incubate the membranes on which 40 antibodies against cytokines were spotted in duplicate (RayBiotech-Tebu Bio, Milano, Italy). The assay was performed according to the manufacturer's instructions. Densitometry was performed by the ImageJ software. Data are expressed as the mean  $\pm$  SD of two separate experiments in duplicate.

*2.4. TransAM assay.* TransAM assays were performed according to the manufacturer's instructions (Active Motif, Carlsbad, CA, USA). TransAM NF $\kappa$ B family kits are ELISA-based kits designed specifically to quantify NF $\kappa$ B activation. In brief, 5  $\mu$ g of nuclear protein samples were incubated for 1 h in a 96-well plate coated with an oligonucleotide that contains the NF $\kappa$ B consensus site (5'-GGGACTTCC-3') to which activated NF $\kappa$ B factors contained in nuclear extracts specifically bind. By using an antibody directed against an epitope on p50 or p65 that is accessible only when NF $\kappa$ B is activated and bound to its target DNA, the NF $\kappa$ B complex bound to the oligonucleotide is detected. After incubation for 1 h with a secondary horseradish peroxidase-conjugated antibody, specific binding was detected by colorimetric estimation on a spectrophotometer at 450 nm. Results are expressed as arbitrary units and represent the mean  $\pm$  SD of two separate experiments in triplicate.

*2.5. Immunoblot analysis and ELISA.* Culture media were resolved by SDS-PAGE, transferred to nitrocellulose sheets at 200 mA for 16 h, and probed with anti-PDGF-BB, anti-RANTES, anti-IL-6, anti-MMP-9 and anti-MMP-2 antibodies (Santa Cruz Biotechnology-Tebu Bio). Secondary antibodies were labeled with horseradish peroxidase (GE Healthcare). The SuperSignal chemiluminescence kit (Pierce, Rockford, IL) was used to detect immunoreactive proteins. In some experiments, nuclear extracts were obtained using the Nuclear Extract kit (Active Motif) and processed by western blot using antibodies against NF $\kappa$ B subunits p50 or p65 (Tebu Bio). All the experiments were repeated at least three times with comparable results.

The amounts of TIMP-2 and IL-8 were measured in 1:2 diluted medium using a double-antibody sandwich ELISA (GE Healthcare) according to the manufacturer's instructions. The concentrations



of TIMP-2 and IL-8 were determined by interpolation from a standard curve and shown as the mean  $\pm$  SD of four separate experiments in triplicates.

*2.6. Gelatin zymography.* Conditioned media were incubated at 4 °C overnight with gelatin–Sephadex. Zymography was performed using 8% polyacrylamide gels co-polymerized with 1 mg/ml gelatin type B (Sigma Aldrich) under non-reducing conditions without heating. Gels were then washed twice for 30 minutes in 2.5% Triton X-100 at room temperature and incubated overnight in collagenase buffer (50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L CaCl<sub>2</sub>, 150 mmol/L NaCl) at 37 °C. Gels were stained in Coomassie Blue R 250 (Bio-Rad, Milano, Italy) in a mixture of methanol:acetic acid:water (4:1:5) for 1 hour and destained in the same solution without the dye. Gelatinase activities were visualized as distinct bands.

*2.7. Statistical Analysis.* Statistical significance was determined using the Student's T test and set at p values less than 0.05. In the figures \*: p < 0.05 \*\*: p < 0.01; \*\*\*: p < 0.001.

### 3. RESULTS

#### 3.1 Activation of NF $\kappa$ B in Mg deficient HUVEC

Because i) Mg deficiency promotes inflammation and atherogenesis [4, 12] and ii) NF $\kappa$ B regulates many genes involved in inflammation and atherosclerosis [13-14], we examined the effects of low Mg on NF $\kappa$ B activation. We first performed EMSA on nuclear extracts prepared from HUVEC cultured in 1.0 or 0.1 mM Mg for 1 h. In nuclear extracts of HUVEC in 1.0 mM Mg, no retarded band was detected whereas two distinct bands could be clearly shown in the samples from cells exposed to 0.1 mM Mg (fig. 1A, left panel). It is noteworthy that these bands were markedly induced by treatment with LPS (1  $\mu$ g/ml), which is used as a positive control, in HUVEC in 0.1 and in 1.0 mM Mg. On the basis of the supershift analysis (fig. 1B), these two bands were identified as the p50/p50 homodimer and p50/p65 heterodimer. Interestingly, in the presence of LPS a third shifted band – by supershift identified as the homodimer p65/65 - was detected. All the bands were specific as assessed by competition assays showing their suppression by incubation with a 50-fold excess of unlabelled NF $\kappa$ B probe (fig 1A, left panel). The bands revealed by EMSA were quantified by densitometry (fig. 1A, right panel).

The localization of p50 and p65, which are known to translocate to the nucleus upon activation, was then assessed by western blot. We found that both p50 and p65 accumulate in the nuclei within 1 h of culture in 0.1 mM Mg (fig. 1C) and that LPS markedly induced the nuclear translocations of the two proteins in HUVEC in 0.1 and in 1.0 mM Mg.

The increase in NF $\kappa$ B binding activity by Mg deficiency correlated with the functional induction of NF $\kappa$ B as demonstrated by reporter gene assays using a vector expressing luciferase under the control of multiple copies of the NF $\kappa$ B responsive consensus. The construct was transiently transfected into HUVEC and the activity of luciferase was monitored as described in the methods. An increase of luciferase activity was observed in cells exposed to 0.1 mM Mg both under basal conditions and after exposure to LPS, indicating that the NF $\kappa$ B complexes are transcriptionally active (fig. 1D).

#### 3.2. Inhibition of NF $\kappa$ B activation by trolox in Mg deficient HUVEC.

Mg deficiency has been associated with oxidative stress [15] which is known to activate NF $\kappa$ B [14]. We therefore evaluated whether an anti-oxidant might prevent NF $\kappa$ B activation in HUVEC cultured in low Mg. Among many anti-oxidants available, for these studies we selected trolox, a water-soluble analog of  $\alpha$ -tocopherol which has been demonstrated to exert a more potent cytoprotective effect than  $\alpha$ -tocopherol [16]. HUVEC were pretreated with trolox (40  $\mu$ M) for 60 min before being exposed to 0.1 mM Mg containing medium for 1 h.

We first evaluated nuclear translocation of p65 and p50 in HUVEC cultured in 0.1 or 1.0 mM Mg in the presence or in the absence of trolox. We found that trolox inhibited p65 and p50 nuclear accumulation (fig. 2A). Quantification through the TransAM assay confirmed this finding for both p50 and p65 (fig. 2B).

These results strongly suggest that NF $\kappa$ B activation by Mg deficiency is mediated by oxidant species.

### *3.3 Modulation of molecules involved in atherogenesis and inflammation in Mg deficient HUVEC.*

We utilized a protein array tailored for proteins involved in inflammation and atherogenesis to study how culture of HUVEC in low Mg modulated the levels of these molecules. To this purpose, HUVEC were cultured for 48 h in 0.1 or 1.0 mM Mg. The medium was then collected and utilized for the proteomic analysis. The results were quantified by densitometry. Figure 3 shows a significant increase of the secretion of PDGF-BB, TIMP-2 and IL-8, and to a lower extent, of granulocyte macrophage-colony stimulating factor (GM-CSF) and RANTES in HUVEC in 0.1 mM Mg. No modulation of secreted transforming growth factor  $\beta$ 1, IL-6 and tumor necrosis factor (TNF)  $\alpha$  was detected. On the basis of their relevance in atherosclerosis, some of these proteins were further investigated by western blot or ELISA. Western blot confirms that, while IL-6 is not altered, secreted PDGF-BB and RANTES are increased in Mg deficient HUVEC (fig. 4, left panel). Figure 4 (right panel) also shows the results of the densitometric analysis. We then measured the levels of IL-8 and TIMP-2 by ELISA. Figures 5A and B show the increase of the two secreted proteins in Mg deficient HUVEC vs controls.

### *3.4 MMP activity in Mg deficient HUVEC.*

Because TIMP-2 antagonizes the action of MMP-2 and -9 [17], we evaluated the effect of Mg deficiency on the expression and on the activity of these proteases. By western blot we found that HUVEC in 0.1 mM Mg secreted higher amounts of the gelatinases MMP-9 and MMP-2 in respect to the controls (fig. 6A). We then analyzed medium collected from HUVEC cultured for 48 h in 0.1 or 1.0 mM Mg by zymography. Two clear bands of gelatinolytic activity, sized to 88 and 68 kDa, corresponding to activated MMP-9 and -2 respectively, were detected. Figure 6B shows the higher gelatinolytic activity of MMP-2 and -9 in the medium from cell cultured in 0.1 mM vs 1.0 mM Mg.

#### 4. DISCUSSION

Mg is the fourth most abundant cation in the human body. Approximately 60-65% of the total body Mg is mineralized in bone, whereas the remaining part is mainly localized in the intracellular compartments and only 1% is circulating in the blood. Current dietary guidelines recommend adequate intake of Mg (310–420 mg daily) to maintain a proper serum Mg concentration without drastically depleting the Mg stores. Hypomagnesaemia (serum Mg concentration below 0.74 mmol/L, or 1.8 mg/dL) can be the result of some therapeutic regimens and of various pathological conditions, including hereditary diseases [8, 18-20]. In rodents, serum Mg concentration drops to 0.1 mM after 8 days on a Mg deficient diet. This is the concentration of extracellular Mg we used in our experimental model because it allows to detect significant effects *in vitro*. It should be pointed out, however, that we previously observed endothelial dysfunction also after exposure to a 0.5 mM Mg containing medium [9]. On the other hand, it is possible that locally restricted dramatic changes in Mg concentration occur and influence endothelial cells in a specific district.

Mg is essential to maintain health and lower the risk of cardiovascular disease. Recent evidence from animal and clinical studies suggests that low Mg is associated with inflammatory processes and atherogenesis [21]. Being endothelial dysfunction a critical step in atherogenesis, it is noteworthy that low Mg induces IL-1, VCAM and PAI-1 and promotes a senescent phenotype in cultured endothelial cells [9-10]. However, the molecular mechanisms involved have not been elucidated. We anticipated a role of the transcription factor NFκB, crucial regulator of inflammation and atherosclerosis [13-14], which is activated in experimental and human atherosclerosis [22]. In Mg deficient HUVEC NFκB is rapidly activated. In particular, we have analyzed the prototypical NFκB complex demonstrating the involvement of p50 and p65 in low Mg dependent NFκB activation. Our results are in agreement with data obtained in primary cultured cerebral vascular smooth muscle cells demonstrating that low extracellular Mg activates NFκB [23]. In addition, Mg supplementation markedly attenuates NFκB nuclear translocation and protects IκB from degradation in LPS-treated endothelial cells [24]. Interestingly, in a model of cyclosporine-induced nephrotoxicity in rats, Mg supplementation showed marked beneficial effects by preventing NFκB activation which is responsible for renal damage [25]. Therefore, Mg levels seem to participate to the complex regulation of NFκB activity in various systems. Mg deficiency has been shown to induce oxidative stress in endothelial cells [15] and oxidative stress is implicated in NFκB activation [22]. In atherogenesis, oxidative stress mediates signalling pathways underlying vascular inflammation, starting from the initiation of fatty streak development, through lesion progression, to plaque rupture [22]. Our results suggest that oxidant species are implicated in NFκB activation, at least early after exposure to low Mg containing medium. Indeed, in Mg deficient HUVEC, the anti-

oxidant trolox, a synthetic cell-permeable analogue of  $\alpha$ -tocopherol, prevents p50 and p65 nuclear translocation and activation. We therefore propose that in Mg deficient HUVEC oxidative stress might activate NF $\kappa$ B and, therefore, trigger NF $\kappa$ B signalling pathway. Accordingly, in HUVEC grown in 0.1 mM Mg we have shown the upregulation of IL-1 $\alpha$ , a pro-inflammatory cytokine target of NF $\kappa$ B, which mediates some of low Mg effects on these cells [9]. In the present study we utilized protein array to obtain a broad profile of the cytokines and growth factors released by Mg deficient HUVEC. Indeed, atherosclerotic plaque development involves several synchronized events such as adhesion, migration and proliferation of cells, all mediated by growth factors and cytokines, some of which resulted significantly increased in Mg-deficient HUVEC. In particular, in cells cultured in low Mg we describe the induction of RANTES, IL-8 and PDGF-BB, all targets of NF $\kappa$ B [22, 26]. A great attention has been focused on PDGF-BB in the origin of atherosclerosis, because of its mitogenic and chemotactic effects on vascular smooth muscle cells [27]. Indeed, the abnormal proliferation and migration of these cells are major processes in the development of vascular disease, not only in atherosclerosis but also in restenosis after angioplasty. PDGF-BB is upregulated by pro-inflammatory stimuli from the early stages of atherogenesis and PDGF-BB mRNA expression is increased in atherosclerotic lesions [28-29].

We also show an increase of the secretion of the chemokines RANTES and IL-8 in Mg-deficient HUVEC. Interestingly, chemokines are important actors in the progression of atherosclerosis as well as in plaque destabilization [30]. In particular, the enhanced expression of IL-8 and RANTES within human atherosclerotic lesions has been reported [22, 30]. IL-8 is critical for chemotaxis and adhesion of monocytes to the endothelial cells, pivotal steps in atherogenesis, and also promotes vascular smooth muscle cell proliferation and migration, crucial events for the progression of the plaque [30]. Similarly, RANTES directs leukocyte attraction and is also involved in neointima formation in atherosclerosis-prone mice. Hence, it is not surprising that antagonizing RANTES reduces atherosclerotic lesion formation and stabilizes the plaque [30]. We therefore conclude that low Mg contributes to atherosclerosis by stimulating endothelial secretion of IL-8, RANTES and PDGF-BB. In our experimental conditions, however, we did not observe any modulation of TNF $\alpha$  and IL-6 which have been shown increased in animal model fed with a Mg-restricted diet [12]. We hypothesize that different cell types, and not endothelial cells, might contribute to secrete these cytokines *in vivo*. Protein array also revealed the increase of GM-CSF, another cytokine which is transcriptionally dependent upon NF $\kappa$ B activation. This finding is puzzling because GM-CSF regulates intimal cell proliferation in the early step of atherosclerosis [31]. More studies are necessary to confirm the regulation of GM-CSF levels in HUVEC cultured in low Mg.

In atherosclerotic plaques, also the expression of MMPs, which degrade collagen fibrils leading to the loss of fibrous cap integrity, is increased [22]. Dysregulated extracellular matrix metabolism has a role in vascular remodelling during the development and complication of human atherosclerotic lesions. The expression of these proteases is normally tightly regulated at the transcriptional level. A second level of control is represented by the requirement of extracellular activation of the latent pro-enzyme. A further level of control of MMP activity involves the irreversible binding of MMPs to the specific tissue inhibitors TIMPs. We found that Mg deficient HUVEC secreted increased amounts of MMP-2 and -9 and of their inhibitor TIMP-2. We anticipated that the increased amounts of TIMP-2 might represent a mechanism to counterbalance MMP activity. However, by zymography we show that MMP-2 and -9 activity overrides the inhibitory effect of TIMP-2. We therefore conclude that MMP/TIMP balance is shifted toward greater MMP activity in Mg-deficient HUVEC. This result further highlights how Mg deficiency might contribute to atherosclerosis. Indeed, an increased expression of MMPs in human atherosclerotic plaques and, in particular, a marked upregulation of MMP-9 in unstable carotid plaque have been demonstrated [32]. Recently, genetic variation within the MMP-2 promoter region was associated with cap thickness and therefore it may influence the role of MMP-2 in plaque vulnerability [33].

## 5. CONCLUSION

Our data indicate that low Mg promotes endothelial dysfunction by activating NFkB which, in turn, triggers a transcriptional program leading to the acquisition of a pro-inflammatory and pro-atherogenic phenotype. Based on epidemiological, cellular and molecular evidences, we propose that broadly correcting nutritional intakes of Mg might represent a simple and inexpensive solution that contributes to the prevention of atherosclerosis.

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## 7. LEGENDS TO THE FIGURES

Figure 1. Induction of NF $\kappa$ B activity in HUVEC cultured in low Mg.

(A) EMSA of the NF $\kappa$ B DNA binding activity was performed on nuclear extracts from HUVEC cultured in 0.1 or 1.0 mM Mg for 1 h, in the presence or in the absence of LPS (left panel). SP and UN are the specific and unspecific competition, respectively, of the LPS-treated HUVEC in 1.0 mM Mg. The binding activity of the constitutively expressed transcription factor oct-1 was used to assess equal loading. Right panel: each band detected by EMSA was quantified by densitometry and expressed on an arbitrary scale.

(B) HUVEC were exposed to LPS for 1 h. Nuclear extracts were incubated with 1  $\mu$ g of anti-p65 or anti-p50 antibodies and then electrophoresed. The shifting of the different NF- $\kappa$ B subunits is indicated by white arrowheads.

(C) Nuclear extracts from HUVEC cultured in medium containing 0.1 or 1.0 mM Mg in the presence or in the absence of LPS were analyzed by western blot using antibodies against p65 or p50. TBP is used as a nuclear marker and GAPDH as an indicator of contamination by cytosol.

(D) Luciferase assay was performed in HUVEC cultured in 0.1 or 1.0 mM Mg as described in the methods. Luciferase activity was detected by fluorimetry. Results are shown as the mean  $\pm$  SD of three separate experiments.

Figure 2. Inhibition of low Mg-induced NF $\kappa$ B activation by trolox.

(A) Nuclear extracts of HUVEC in 0.1 or 1.0 mM Mg in the presence or in the absence of trolox (40  $\mu$ M) for 1 h were obtained. Western blot was performed with antibodies against p65 or p50. TBP is shown as loading and fraction purity control. GAPDH shows that cytosolic contamination is very low.

(B) p65 and p50 activity was quantified by TransAM NF $\kappa$ B analysis on nuclear extracts of HUVEC cultured in 0.1 or 1.0 mM Mg with or without trolox. Results are expressed as arbitrary units and represent the mean  $\pm$  SD of 3 separate experiments.

Figure 3. Protein arrays on media collected from HUVEC cultured in 0.1 or 1.0 mM Mg for 48 h. Densitometric analysis on array spots was performed and data are expressed as the mean  $\pm$  SD.

Figure 4. Amounts of secreted RANTES, PDGF-BB and IL-6 in Mg deficient HUVEC. A representative western blot on conditioned media from HUVEC cultured in 0.1 or 1.0 mM Mg is



shown (left panel). Densitometric analysis was performed and data are expressed as the mean  $\pm$  SD of three separate experiments (right panel).

Figure 5. Increased secretion of IL-8 and TIMP-2 in Mg deficient HUVEC.

(A) IL-8 and (B) TIMP-2 were detected by ELISA in media collected from HUVEC cultured in 0.1 or 1.0 mM Mg. Data are expressed as the mean of 3 different experiments in triplicate  $\pm$  SD.

Figure 6. Increased secretion of MMP-2 and -9 in Mg deficient HUVEC.

Representative western blot (A) and zymography (B) on media collected from cells cultured in 0.1 or 1.0 mM Mg are shown.

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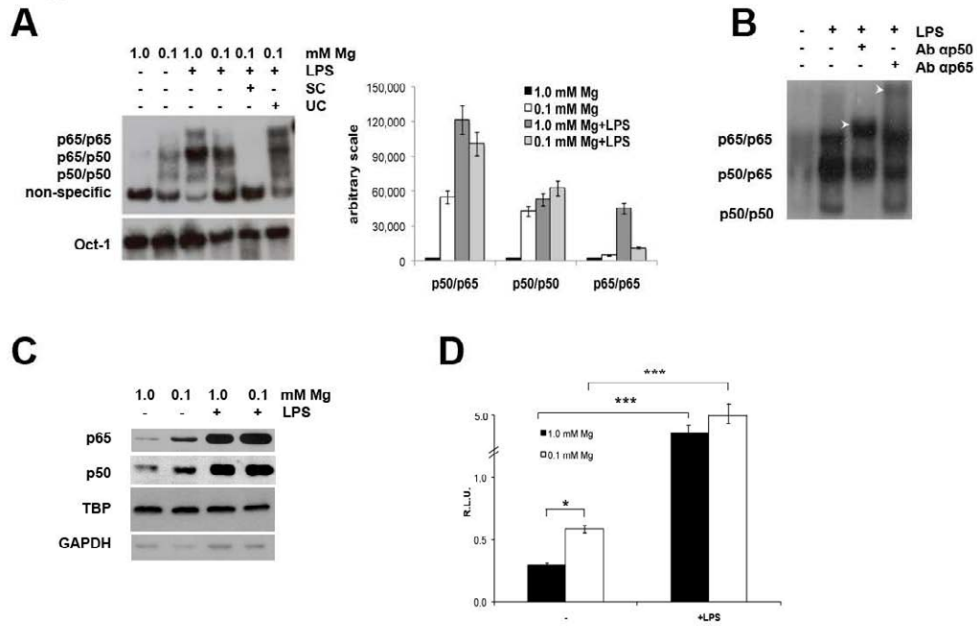
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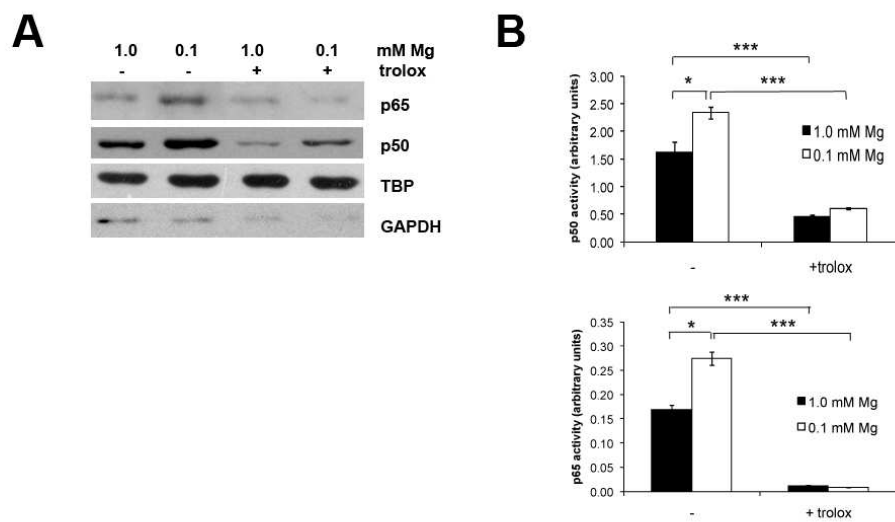
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Fig.1

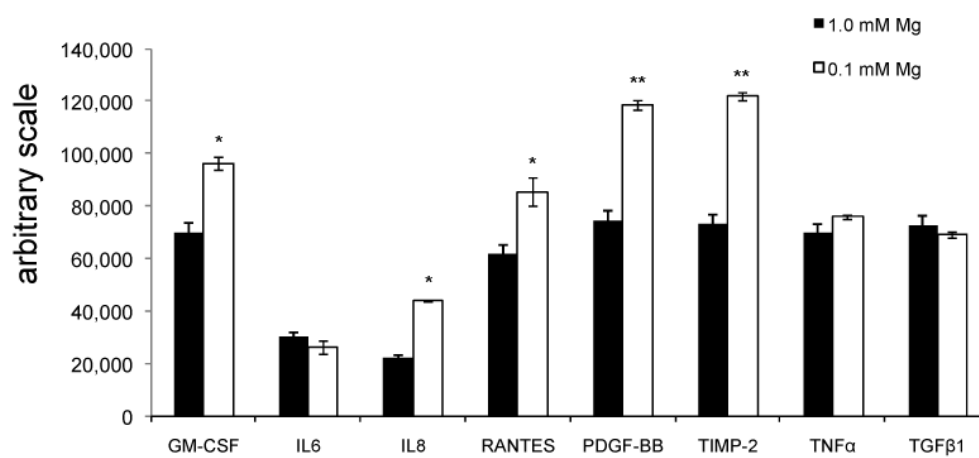


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Fig. 2

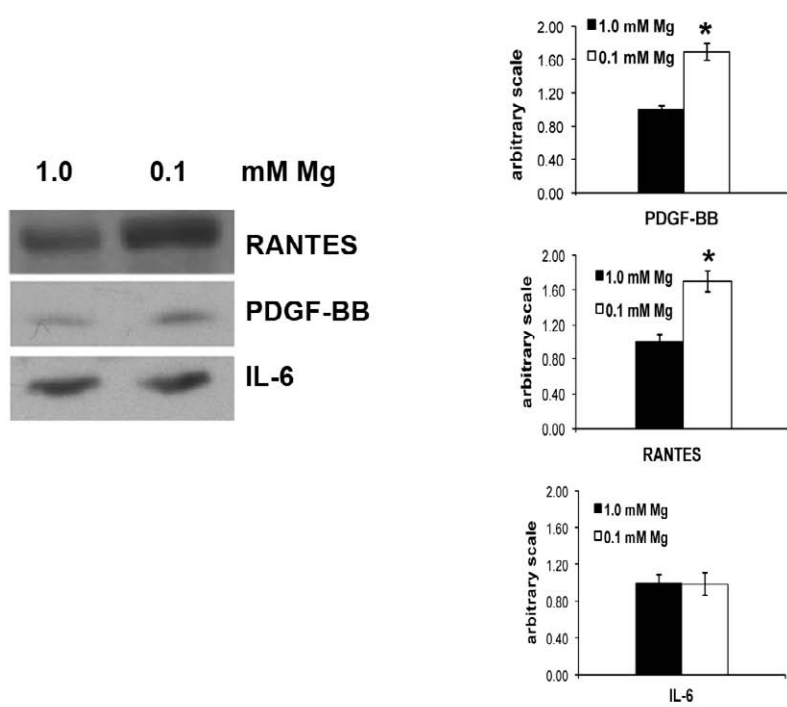


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**Fig. 3**

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Fig. 4



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Fig. 5

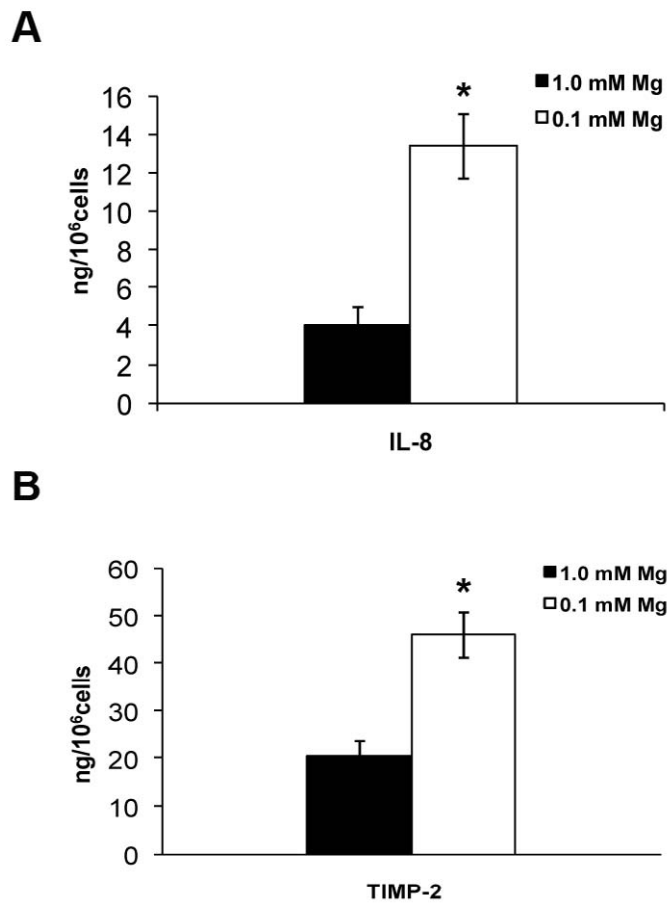
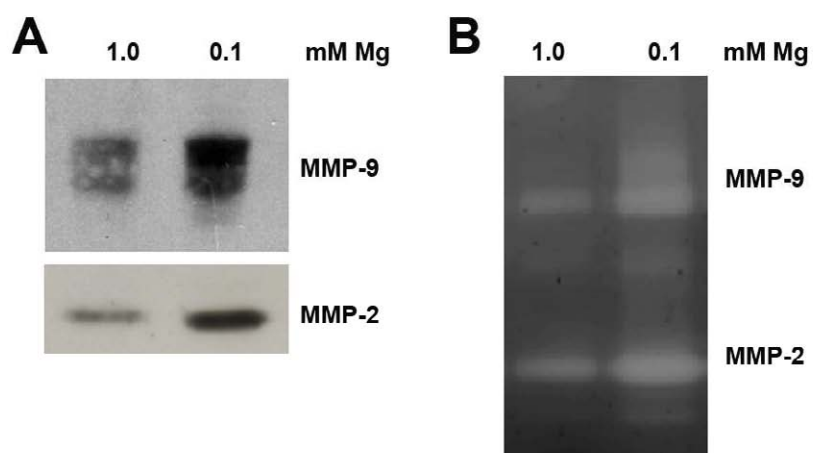


Fig. 6



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