4. RESULTS-1

Mg in macrovascular endothelial cells.

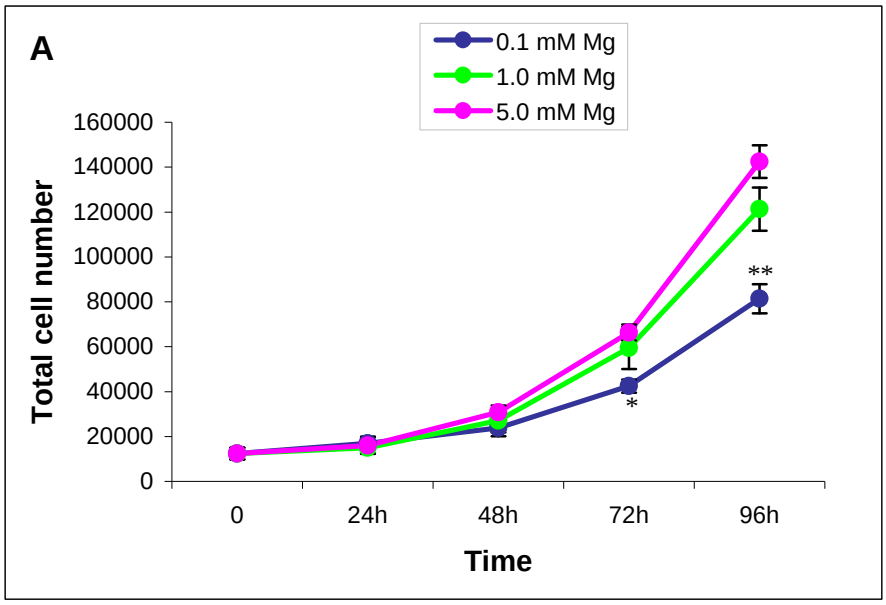
4.1 Mg MODULATES THE ENDOTHELIAL PROLIFERATION

Endothelial cells are essential to maintain the integrity of the vascular wall. They are crucial in the control of permeability, in the modulation of blood flow and in the regulation of inflammatory response (Cines DB et al. 1998).

It is known that endothelial dysfunction plays a pathogenic role in several diseases, including atherosclerosis, thrombosis and hypertension (Ross R., 1999). Because i) Mg deficiency is one of the factors involved in the pathogenesis of cardiovascular disease in general and of atherosclerosis in particular (Shechter M., 2010), and ii) Mg plays a crucial role in cell proliferation and function (Wolf F. and Trapani V., 2008), I studied the effects of different Mg concentration on the growth of endothelial cells isolated from human umbilical vein endothelial cells (HUVEC), which are widely considered as a model of macrovascular endothelial cells.

HUVEC were cultured in the presence of different concentrations of extracellular Mg (0.1, 1.0 and 5.0 mM) for 4 days and counted every 24 hours. The 1.0 mM Mg concentration is considered the physiological concentration of the cation, the 0.1mM Mg is a concentration used to mimic severe Mg deficiency. In humans, the lowest level of Mg in critically ill subjects was 0.5 mM while concentrations ranging between 0.7 and 0.8 mM are frequent in apparently healthy western people. In rodents, after 8 days of experimental diet, a decrease in plasma Mg to a 0.14 mM can be detected. On the other hand, 3-5 mM is the concentrations of plasma Mg that could be reached locally, after acute Mg administration and severe hypermagnesemia has been observed in chronic renal failure and in dialysized patients (Navarro-Gonzales JF, 2009). In particular, 5mM has been previously utilized *in vitro* in different cells (Maier JAM et al., 2004; Wolf F. and Cittadini A. et al., 1999).

As shown in Figure 4.1A, Mg modulates endothelial proliferation in a dose dependent manner. Culture in low Mg inhibited HUVEC cell proliferation. This growth inhibition was reversible upon return to normal culture conditions after re-addition of Mg to the medium (Figure 4.1B) and was independent from the Mg salt used for supplementation ($MgSO_4$, $MgCl_2$ or MgPidolate).



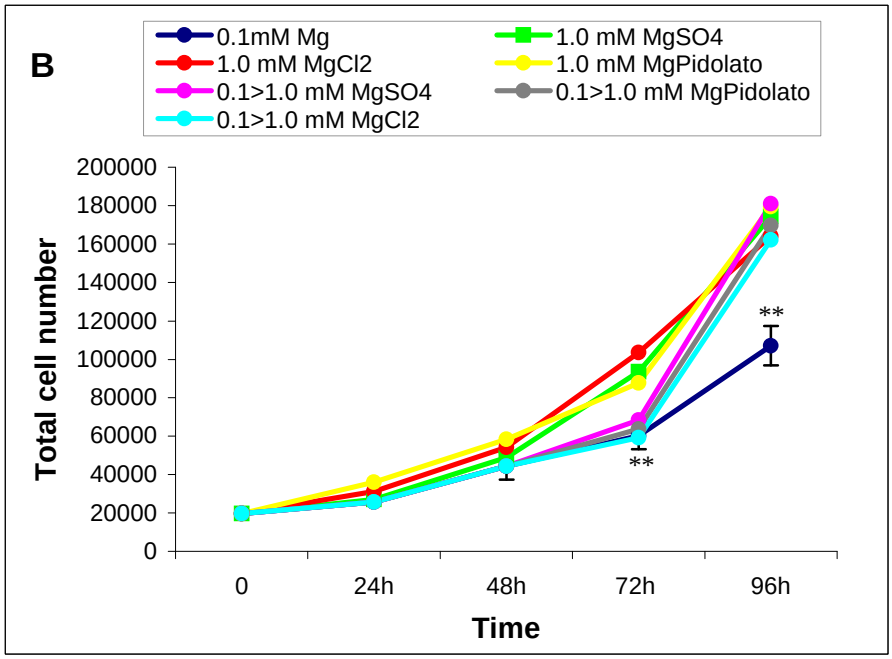


Figure 4.1: Reversible inhibition of endothelial proliferation by low Mg.

A) HUVEC were seeded at low density and cultured in medium with different Mg concentrations. At different intervals of time (every 24 hours) the cells were tripsinized and viable were counted with Burker chamber. Results are expressed as mean ± S.D. of three separate experiments. Significant difference are indicated as a p value less than 0.01 (** $p<0.01$).

B) HUVEC were cultured in media containing 0.1 and 1.0 mM Mg with different Mg salts. Every 24h the cells were harvasted with trypsin and viable counted using a Burker chamber. After 48h, some of the cells in 0.1 mM Mg were reverted to 1.0 mM Mg for additional 48h. The figure shows the results of three different experiments in triplicate ± S.D. (** $p<0.01$).

In an effort to gain more insights into the inhibition of HUVEC growth by 0.1 mM Mg, cell cycle distribution was evaluated. HUVEC cultured in low Mg showed an increased number of cells in the G0/G1 and G2/M phases and a decreased number of cells in the S phase of the cell cycle (Figure 4.2A). Growth inhibition by low Mg correlated with a 5 fold increase of p21 (WAF1), an inhibitor of cyclin-dependent kinase, and a 9 fold reduction of Cdc25B, a phosphatase which plays a key role in controlling G2-M progression (Nilsson I. and Hoffmann I., 2000), as detected by western blot followed by densitometry (Figure 4.2B).

Concentration of extracellular Mg [mM]	Distribution (%)			
	Apoptosis	G0/G1	S	G2/M
1.0	13.0 ± 2.2	50.1 ± 4.5	46.9 ± 4.0	2.0 ± 0.3
0.1	15.1 ± 2.3	58.5 ± 4.8	35.3 ± 3.3	7.2 ± 0.6

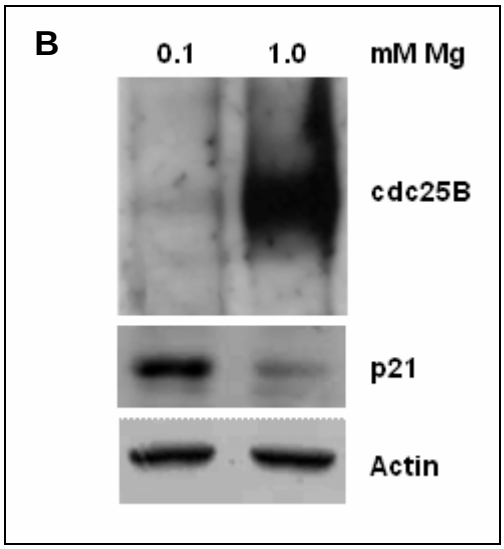


Figure 4.2:

A) Cell cycle distribution in HUVEC grown 0.1 or 1.0 mM Mg as detected by flow cytometry on propidium iodide-stained cells. Data are mean ± SD of three separate experiments.

B) Cdc25B and p21 levels in HUVEC grown 0.1 or 1.0 mM Mg. Cell extracts from HUVEC cultured in 0.1 or 1.0 mM Mg for 72 h were separated on a 12% SDS-PAGE, transferred to nitrocellulose and western blot was performed using anti-Cdc25B and anti-p21 antibodies. Actin is used as a control of loading.

4.2 LOW Mg INDUCES INTRACELLULAR REACTIVE OXYGEN SPECIES PRODUCTION

Mg deficiency has been associated with oxidative stress (Mazur et al., 2007), a crucial event in aging, atherosclerosis and other cardiovascular diseases (Bono F. et al., 2008; Donato AJ et al., 2007). Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical are produced continuously in cells as products of the metabolism and cellular redox processes. During the inflammatory response, activated leukocytes release ROS which alter vascular endothelial function. In response to inflammation, also endothelial cells produce more free radicals. Indeed, in atherogenesis, endothelial cells are primarily involved in oxidating LDL (Libby P et al., 2009). Under physiological conditions, free radicals are countered by antioxidant enzymes and substances. Excessive production of free radicals leads to structural and functional damage of the lipids, proteins and nucleic acids.

To investigate whether Mg deficiency induces early ROS production in culture macrovacular endothelial cells, we used a fluorescent probe and measured the fluorescence emitted by 2,7-dichlorofluorescein (DCF). HUVEC were cultured in 0.1 or 1.0 mM Mg for 1h. The cells were then incubated with DCF (25μM) for 30 min and exposed or not to H_2O_2 (200μM). The fluorescence was measured with the PerkinElmer TopCount Microplate fluorimeter. The basal level the ROS production in cells cultured in 0.1 or 1.0 mM Mg is the same while after incubation with H_2O_2, ROS production is higher in cells cultured in medium with 0.1 mM Mg (Figure 4.3).

These results suggest that Mg deficiency determines a latent impairment of anti-oxidant defences which is unmasked by the addition of H_2O_2.

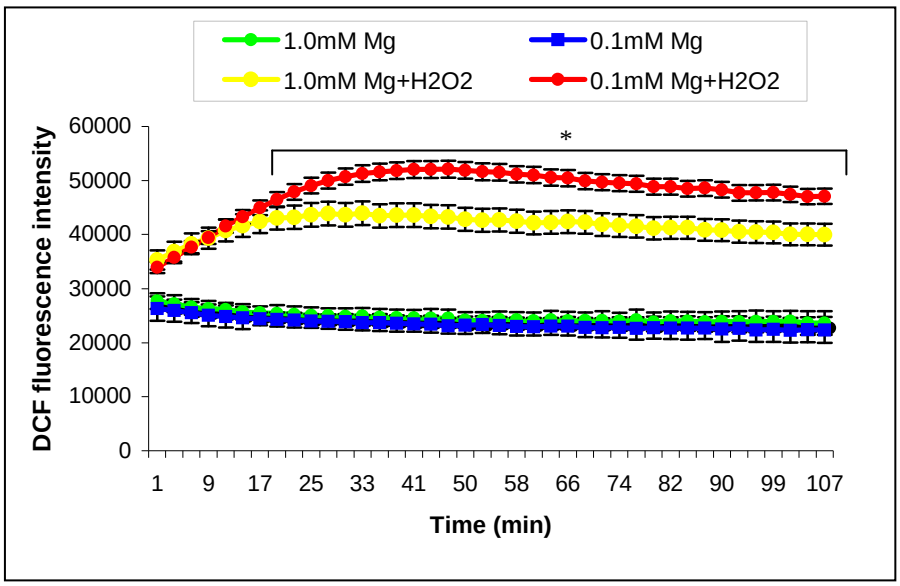


Figura 4.3: Time course of low Mg-induced reactive oxygen species production.

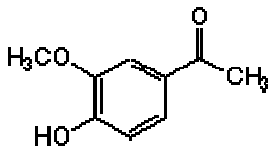
Huvec exposed at medium containing 0.1 or 1.0 mM Mg for 1h were incubated with DCF (25μM) for 30 min and exposed or not to H_2O_2. Cellular oxidant production was determined by PerkinElmer TopCount Microplate fluorimeter. Results are expressed as mean ± S.D. of three separate experiments. Significant difference are indicated as a p value less than 0.05 (* $p<0.05$).

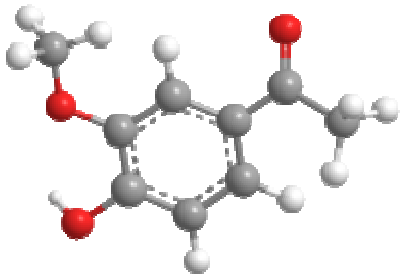
4.3 THE ANTIOXIDANTS APOCYNIN AND TROLOX RESTORE PROLIFERATION IN HUVEC CULTURED IN LOW Mg

Since low Mg promotes an oxidant phenotype, we asked whether antioxidants might affect the proliferation rate of HUVEC in low Mg. Trolox, resveratrol and apocynin were utilized as anti-oxidants. Trolox is a water-soluble analog of α-tocopherol (vitamin E) and protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber Atkinson J, 2007). Resveratrol has been shown to possess cardioprotective and neuroprotective effects. The mechanisms of resveratrol action have not been completely undisclosed, but it is known that it increases the activity of Mn-superoxide dismutases (SOD) by a fourteen-fold (Robb EL et al., 2008). MnSOD is essential for the cell to keep superoxide anions in check.

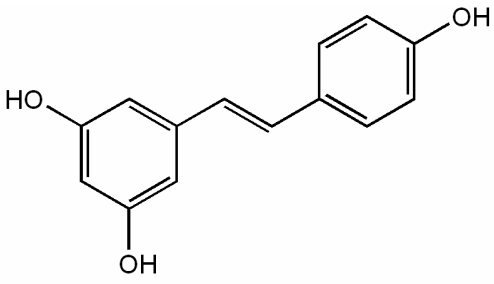
Apocynin is an inhibitor of NADPH oxidase activity, which is the major source of reactive oxygen species in endothelial cells under inflammatory stimuli (Ray R and Shah AM, 2005).

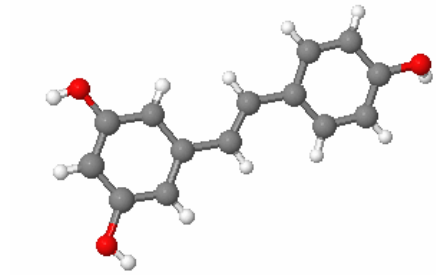
A: apocynin





B: resveratrol





C: α-tocopherol

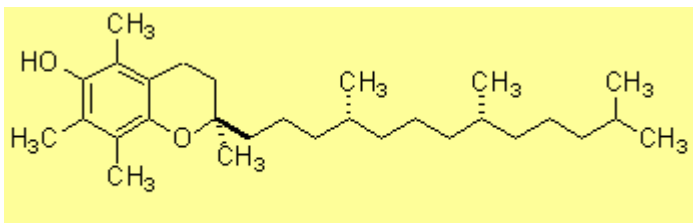


Figure 4.4: Antioxidant chemical structure.

HUVEC in 0.1 or 1.0 mM Mg were cultured in the presence or not of trolox, resveratrol and apocynin. Every 48h, the cells were trypsinized and counted. Figures 4.5A and 4.5B show that trolox and apocynin could rescue the proliferation of cells grown in 0.1 mM Mg. These results indicate that oxidative stress in implicated in braking HUVEC proliferation and point to NADPH oxidase as the principal responsible of oxidative stress in HUVEC cultured in low extracellular concentrations of Mg.

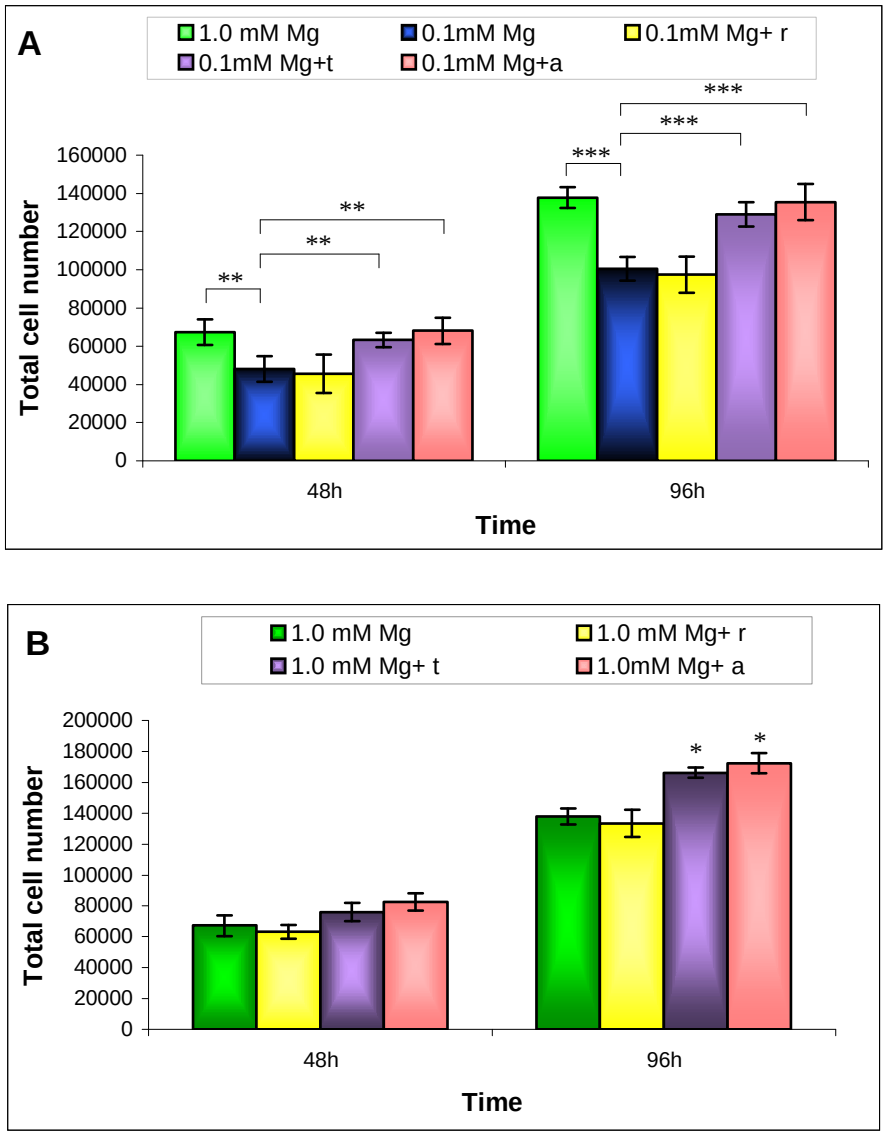


Figure 4.5: Trolox and apocynin restore HUVEC proliferation in low Mg.

(A) HUVEC were seeded at low density and cultured in medium with 0.1mM Mg concentration and with different antioxidant molecules: t: trolox (40µM); a: apocynin (10µM) and r: resveratrol (10µM). At different intervals of time (48 and 96 hours) the cells were tripsinized and viable were counted with Burker chamber. Results are expressed as mean ± S.D. of three separate experiments. Significant difference are indicated as a p value less than 0.01 (** $p<0.01$).

(B) HUVEC were seeded at low density and cultured in medium with 1.0mM Mg concentration and with different antioxidant molecules: t: trolox (40μM); a: apocynin (10μM) and r: resveratrol (10μM). At different intervals of time (48 and 96 hours) the cells were tripsinized and viable were counted with Burker chamber. Results are expressed as mean ± S.D. of three separate experiments. Significant difference are indicated with ** $p<0.01$ and * $p<0.05$.

4.4 ACTIVATION OF NFkB IN Mg DEFICIENT HUVEC

Inflammation is considered to be a critical initial step in the development of atherosclerosis. Pro-inflammatory changes in endothelial phenotype, known as “endothelial activation”, involve upregulation of cellular adhesion molecules with consequent enhancement of endothelial-leucocyte interactions and the increase of permeability, as well as alterations in the secretion of autocrine/paracrine factors, which are pivotal in the inflammatory responses. Because i) Mg deficiency promotes inflammation and atherogenesis in *in vivo* models (Maier JAM, 2004), ii) NFkB orchestrates inflammation (Barnes PJ and Karin M, 1997) and iii) oxidants activate NFkB (Karin M. et al., 2001), the effects of low Mg on NFkB activation was examined in HUVEC. NFkB is a redox-sensitive transcription factor composed of homo- and heterodimeric complexes of members of the Rel(NFkB) family. There are five subunits of the NFkB family in mammals: p50, p65, c-Rel, p52 and RelB. In the majority unstimulated cells, NFkB is maintained in an inactive, IkB-bound complex in the cytoplasm and upon activation of NFkB, IkB is phosphorylated by IkB kinases (IKK) which targets the protein for ubiquitination and degradation by 26S proteasome. Degradation of IkB unmasks the nuclear localization signal of NFkB dimers which translocate to the nucleus where they bind to DNA. NFkB proteins bind DNA motif with the consensus sequence: GGGRNNYYCC, where R= purine, N= any base and Y= pyrimidine. Among various signals inducing NFkB, I recall TNF-α, IL-1, bacterial lipopolysaccharide (LPS), advance glycation products, hyperglycemia, platelet-activation factor, shear stress, oxidized lipids, oxidant stress and hypoxia/reperfusion, all involved in endothelial dysfunction (Pahl HL, 1999) (Figure 4.6).

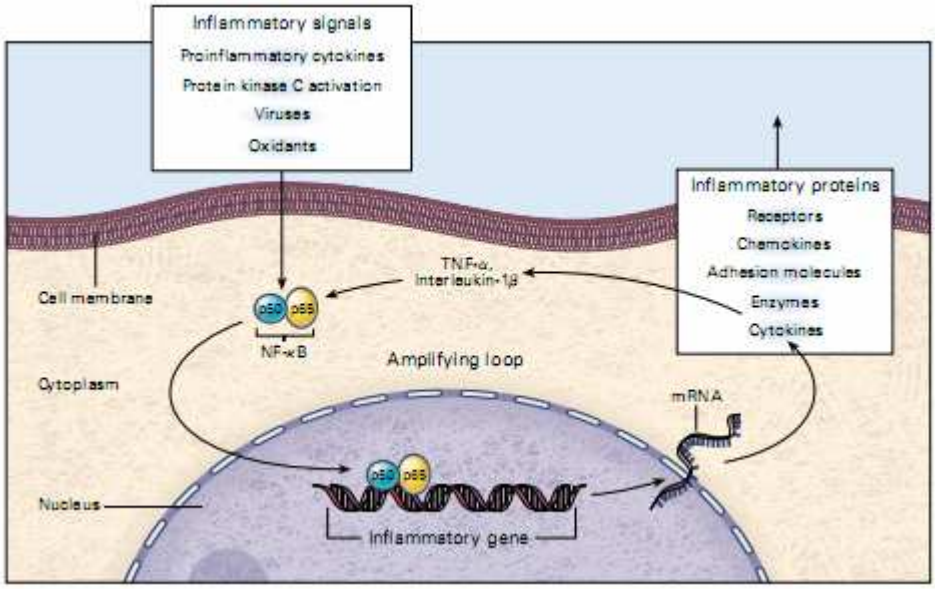


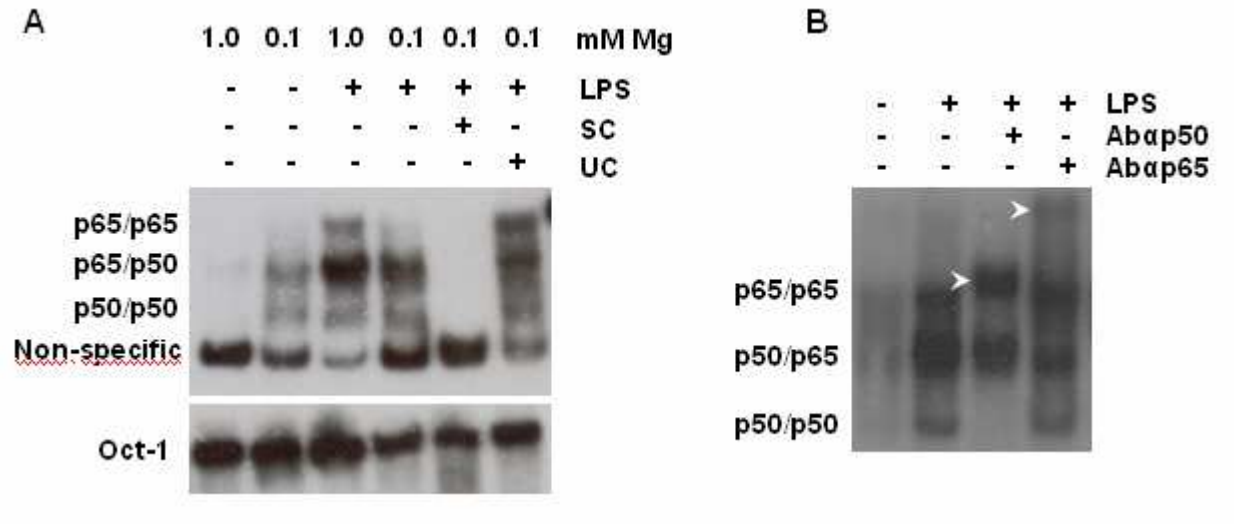
Figure 4.6: NFkB activation (Barnes PJ et al., 1997)

I first performed an Electrophoretic Mobility Shift Assays (EMSA) on nuclear extracts prepared from HUVEC cultured in 1.0 or 1.0 mM Mg for 1h. 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 (Figure 4.7A). It is noteworthy that these bands were markedly induced by treatment with LPS (1µg/ml), which was used as a positive control, in HUVEC in 0.1 and 1.0 mM Mg. On the basis of the supershift analysis (Figure 4.7B), 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/p65 - was detected. All bands were specific as assessed by competition assays showing their suppression by incubation with a 50-fold excess of unlabelled NFkB probe (Figure 4.7A).

The localization of p65 and p50, which are known to translocate to the nucleus upon activation, was then assessed by western blot. I found that both p50 and p65 accumulate in the nuclei within 1h of culture in 0.1 mM Mg (Figure 4.7C) and that LPS markedly induced the nuclear translocation of the two proteins in HUVEC cultured in 0.1 or 1.0 mM Mg.

The increase of NFkB binding activity by Mg deficiency correlated with the functional induction of NFkB as demonstrated by reporter gene assays using a vector containing multiple copies of an NFkB response element that drives transcription of the luciferase reporter gene *luc2P* (*Photinus pyralis*). The construct was transiently transfected into HUVEC and the luciferase activity was measured as described in Matherials and methods. An increase of luciferase activity was observed in cells cultured to 0.1 mM Mg both under basal condition and after exposure to LPS indicating that the NFkB complexes are transcriptionally active (Figure 4.7D).

These experiments demonstrate that Mg deficiency activates NFkB in HUVEC.



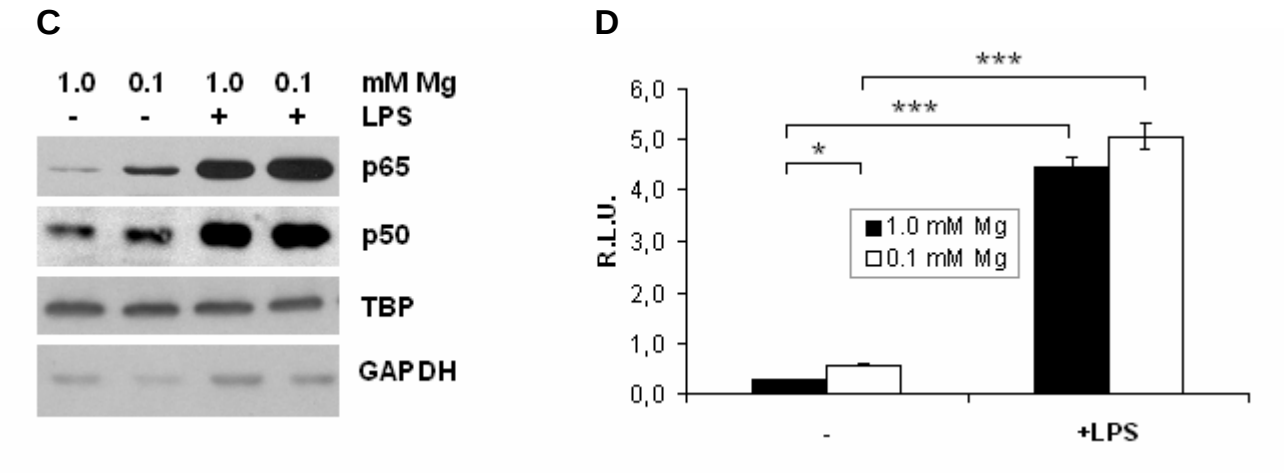


Figure 4.7: Induction of NFkB activity in HUVEC cultured in low Mg.

A) EMSA of the NFkB DNA binding activity was performed on nuclear extracts from HUVEC cultured in 0.1 or 1.0 mM Mg for 1h, in the presence or in absence of LPS. 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.

B) HUVEC were exposed to LPS for 1h. Nuclear extracts were incubated with 1µg of anti-p65 or anti-p50 antibodies and then electrophoreses. The shifting of the different NFkB subunits is indicated by white arrowheads.

C) Nuclear extracts from HUVEC cultured in medium containing 0.1 or 1.0 mM Mg with or without LPS were analyzed by western blot using antibodies against p65 or p50. TBP was used as a nuclear marker and GAPDH as an indicator of contamination by citosol.

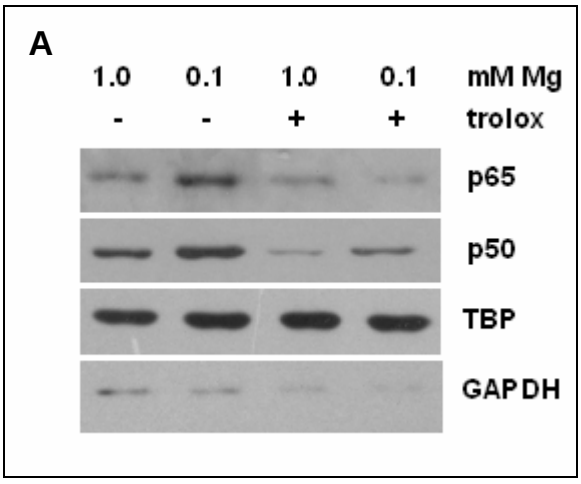
D) Luciferase assay was performed in HUVEC cultured in 0.1 or 1.0 mM Mg as decribed in Materials and methods. Luciferase activity was detected by fluorimetry. Results are shown as the mean ± SD of three separate experiments (* $p< 0.05$ and *** $p< 0.001$).

4.5 INHIBITION OF NFkB ACTIVATION BY TROLOX IN Mg DEFICIENCY

Mg deficiency has been associated with oxidative stress which is known activate NFkB. We therefore evaluated whether an anti-oxidant might prevent NFkB activation in HUVEC cultured in low Mg. Among many anti-oxidants available and tested on HUVEC proliferation, for these studies I selected trolox, a water-soluble analog of α-tocopherol. HUVEC were pretreated with trolox (40 μM) for 60 min before being exposed to 0.1 mM Mg containing medium.

We first evaluated nuclear translocation of p65 and p50 in HUVEC cultured in 0.1 or 1.0 mM Mg with or without trolox by western blot on nuclear extracts. We found that trolox inhibited p65 and p50 nuclear translocation in cells cultured in medium containing 0.1mM Mg (Figure 4.8A). These data were confirmed by two other methods: i) the TransAM assay for both p50 and p65 subunits (Figure 4.8B) and ii) the immunofluorescence with antibody against the p65 subunit (Figure 4.8C). Similar results were obtained also with apocynin (not shown).

These results strongly suggest that NFkB activation by Mg deficiency is mediated by oxidant species.



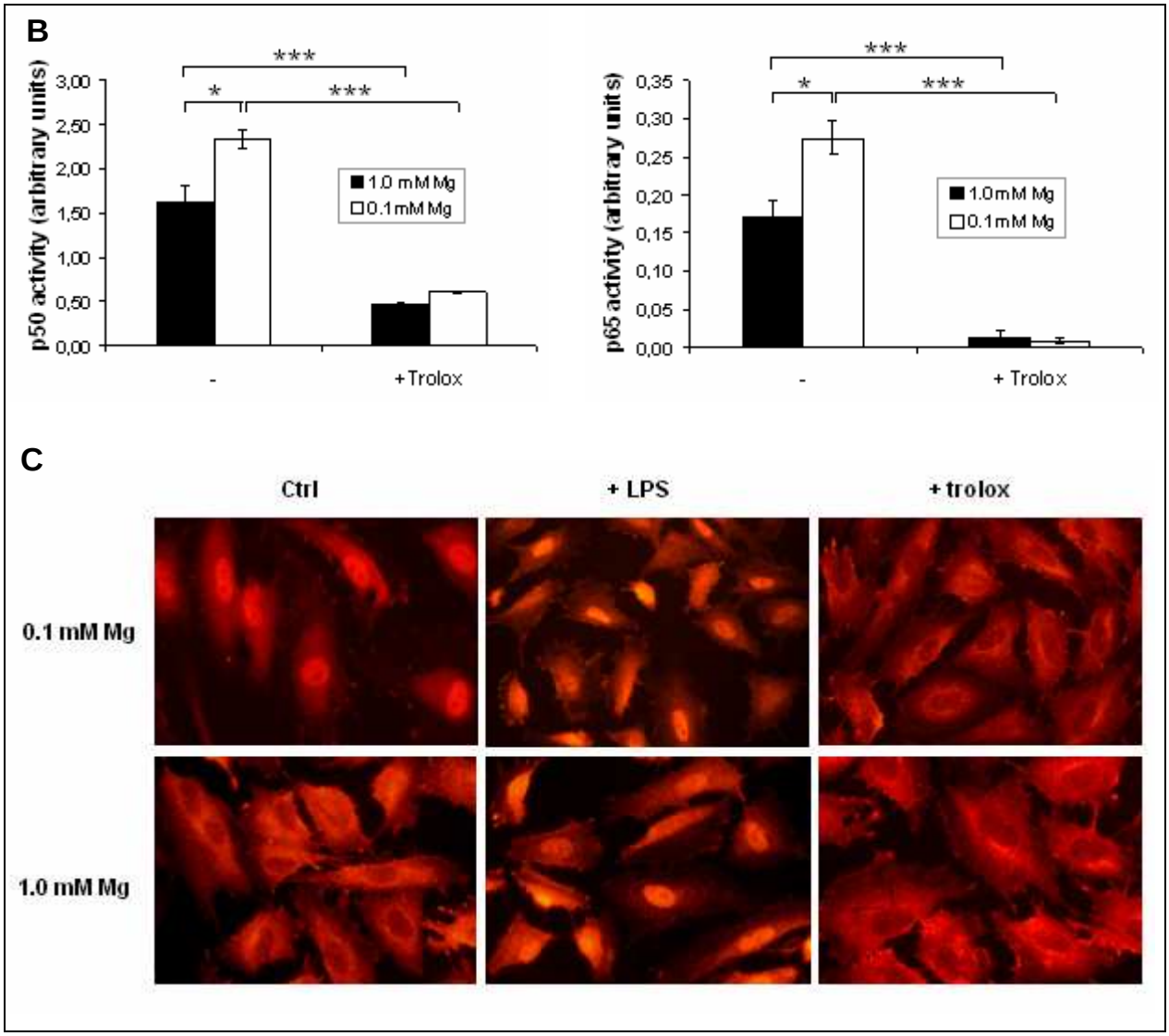


Figure 4.8: Inhibition of low Mg-induced NFkB activation by trolox.

A) Nuclear extracts of HUVEC in 0.1 or 1.0 mM Mg in presence or absence of trolox for 1h were obteined. 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 NFkB 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 ± SD of three separate experiments (* $p<0.05$ and *** $p<0.001$).

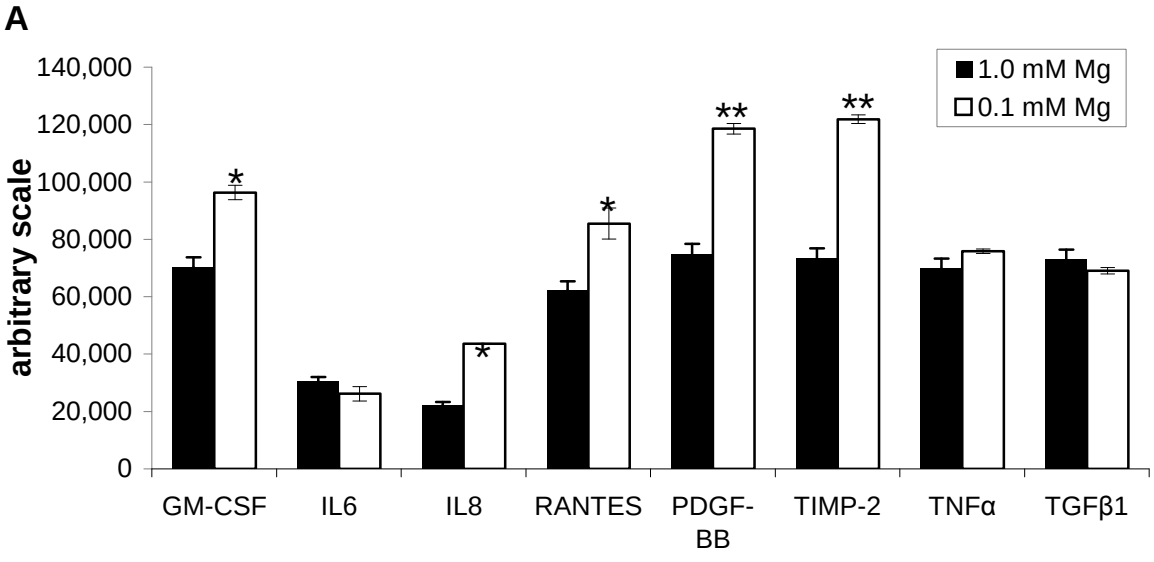
C) HUVEC were cultured on coverslips in medium 0.1 or 1.0 mM Mg for 1h. After the incubation the cells were fixed, stained with antibody against the p65 subunit followed by rhodamine labeled anti-rabbit IgGs and analyzed using a fluorescence microscopy (magnification 40x). **Ctrl**: untreated HUVEC in 1.0 or 0.1 mM Mg; **+ LPS:** HUVEC in 1.0 or 0.1 mM Mg treated for 1 h with LPS; **+ trolox**: HUVEC pretreated with trolox for 1h, followed by culture in 0.1 or 1.0 mM Mg for another 1 h. Three independent experiments with the same results were performed.

4.6 Mg DEFICIENCY MODULATES THE SECRETION OF MOLECULES INVOLVED IN INFLAMMATION AND ATHEROGENESIS

To study whether culture of HUVEC in low Mg modulated the levels of proteins involved in inflammation and atherosclerosis, I utilized a protein array tailored for these molecule.

To this purpose, HUVEC were cultured for 48 h in 0.1 mM or 1.0 mM Mg. The medium was then collected and utilized for the proteomic analysis. The results were then quantified by densitometry. Figure 4.9A 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 (TGF) β1, IL-6 and tumor necrosis factor (TNF) α was detected in cells in 0.1 mM Mg. 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 (Figure 4.9B). I then measured the levels of IL-8 and TIMP-2 by ELISA which confirmed the increase of secreted IL-8 and TIMP-2 in Mg deficient HUVEC vs controls (Figure 4.9C and 4.9D).

Elevated levels of the proinflammatory cytokine interleukin 1α (IL-1) have been detected in rats fed with low Mg diet (Mazur A, 2007). Since IL-1 profoundly affects endothelial behavior and is a potent inhibitor of endothelial cell growth (Maier JAM, 1990), I evaluated the levels of IL-1 in HUVEC cultured in 0.1 or 1.0 mM Mg for 24 and 72 h by ELISA. Fig. 4.8E shows a marked increase of IL-1 in the cell extracts of HUVEC exposed to 0.1 mM Mg.



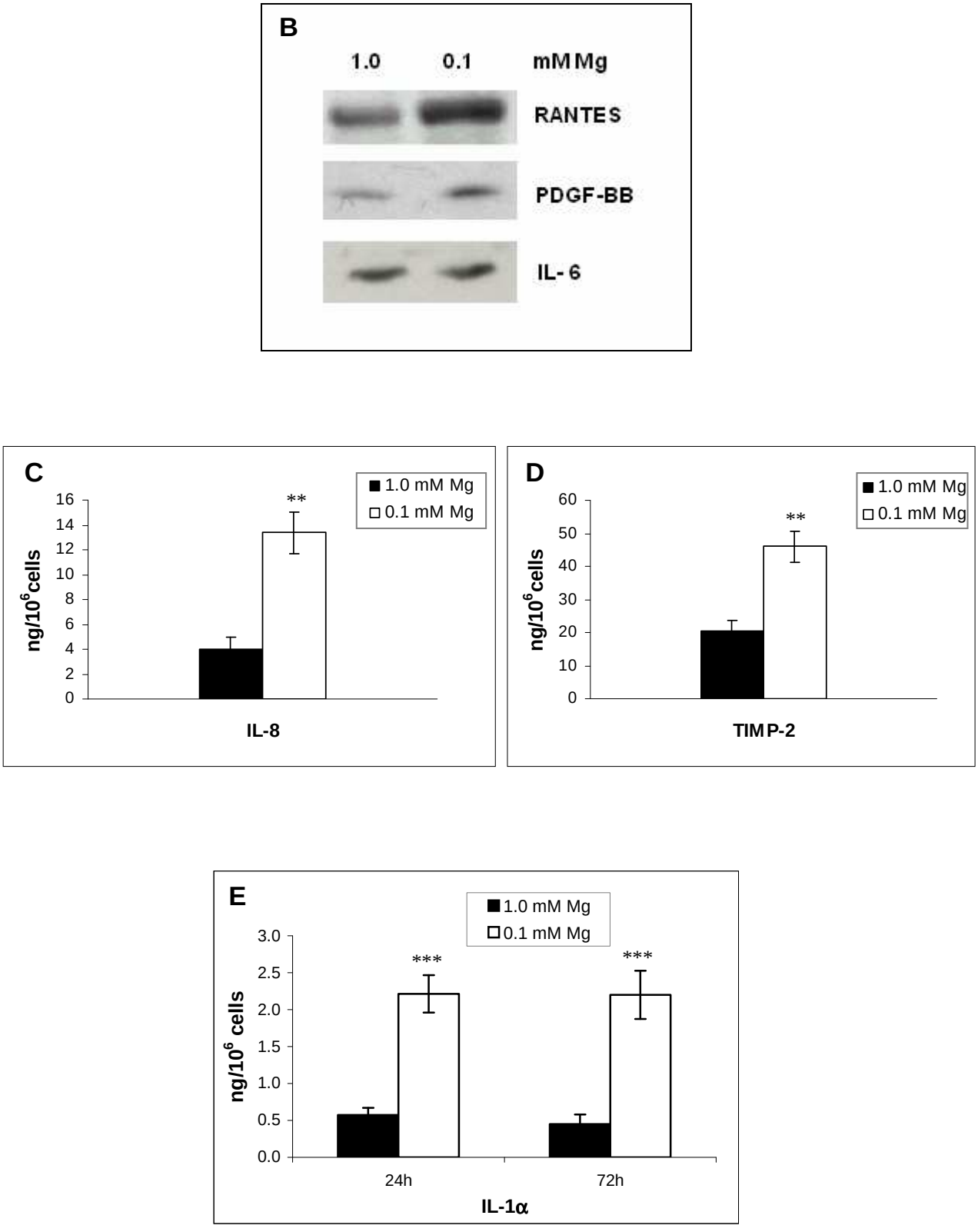


Figura 4.9: Molecules modulate in low Mg cells.

A) Protein array on media collected from HUVEC cultured in 0.1 or 1.0 mM Mg for 48h. Densitometric analysis on array was performed and data are expressed as the mean ± S.D. of two different experiments (* $p<0.05$ and ** $p<0.01$).

B) A representative western blot on conditioned media from HUVEC cultured in 0.1 or 1.0 mM Mg that shows the amounts of secreted RANTES, PDGF-BB and IL-6.

C) D) Increase secretion of IL-8 and TIMP-2 respectively in media collected from HUVEC cultured in low Mg for 48h and detected by ELISA. Data are expressed as the mean of three different experimentsin triplicate ± S.D. (** $p<0.01$).

E) ELISA for IL-1. IL-1 was measured in cell extracts from HUVEC cultured in low Mg for 24h and 72h by ELISA. Data are expressed as the mean of three different experiments in triplicate ± S.D. (*** $p<0.01$).

4.7 MMPs ACTIVITY IN Mg DEFICIENCY HUVEC

Because TIMP-2 antagonizes the action of MMP-2 and -9, we evaluated the effect of Mg deficiency on the amounts and on the activity of these proteases. By western blot I found that HUVEC in 0.1 mM Mg secreted higher amounts of the gelatinases MMP-9 and MMP-2 in respect to the controls (Figure 4.10A). I then analyzed medium collected from HUVEC cultured for 72 h in 0.1 or 1.0 mM Mg by zymography. Two clear bands of gelatinolytic activity, sized to 92 and 62 kDa, corresponding to activated MMP-9 and -2 respectively, were detected. Figure 4.10B shows the higher gelatinolytic activity of MMP-2 and -9 in the medium from cells cultured in 0.1 mM vs 1.0 mM Mg.

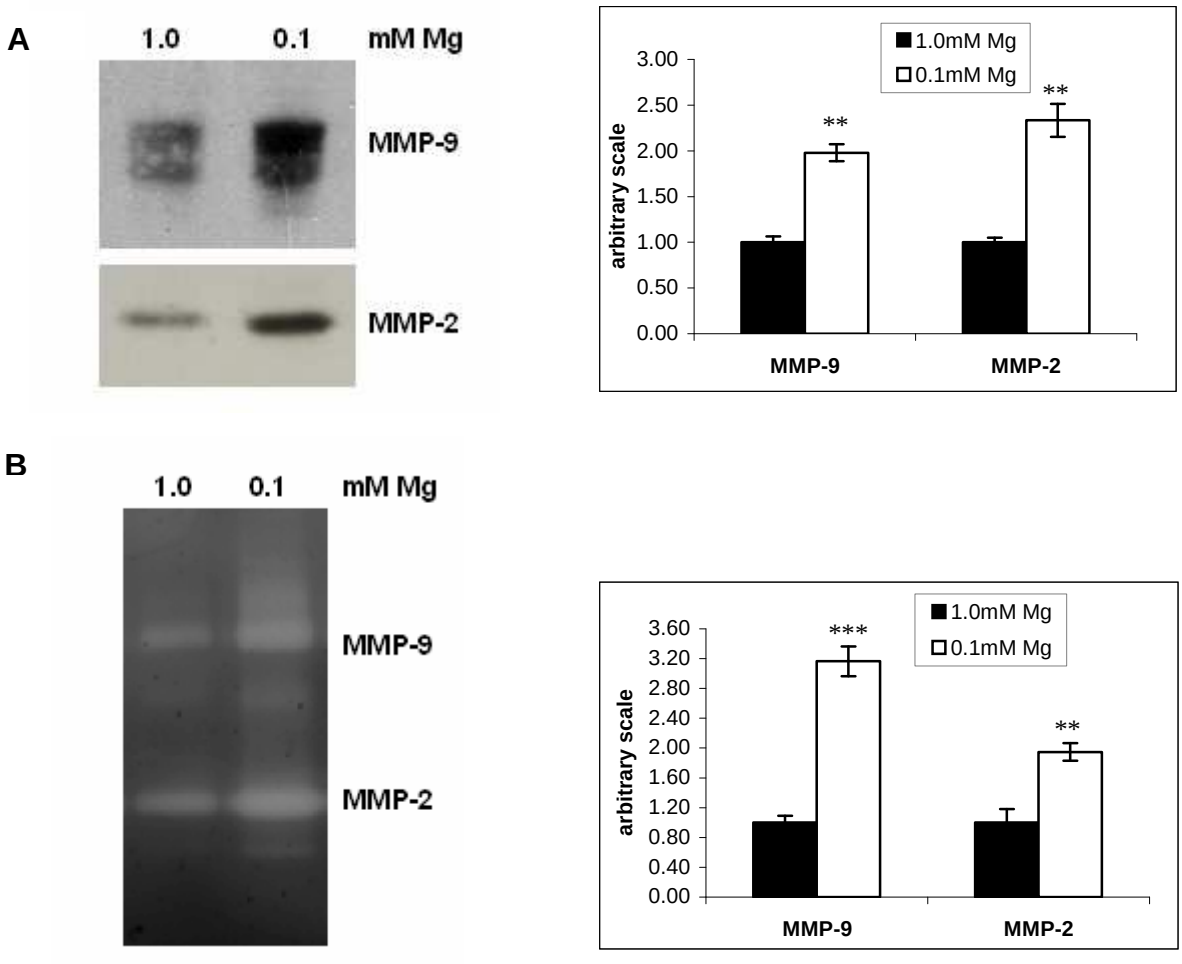


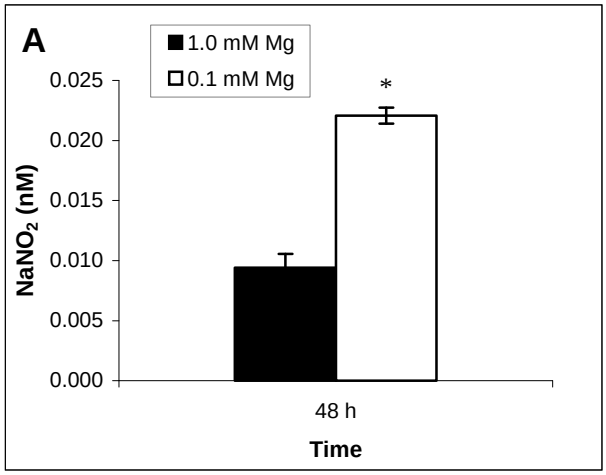
Figura 4.10: Effect of low Mg on MMP secretion.

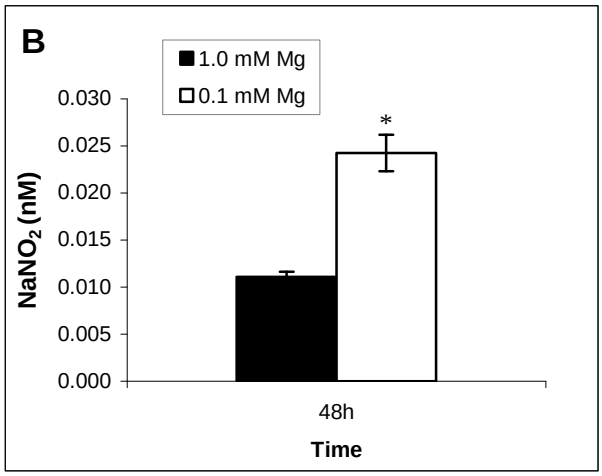
A) Representative western blot and **B)** zymography were performed on culture media from cells cultured in 0.1 or 1.0mM Mg as described in Materials and Methods.
The histograms show the quantitative evaluation of MMP-9 and MMP-2 amounts (western blot) or activity (zymography) in 0.1 mM Mg compared to 1.0 mM Mg by densitometry. Significant increases in MMP-9 and MMP-2 are shown as a p value less than 0.01 (** $p<0.01$) and 0.001 (*** $p<0.001$). Data are representative of three separate experiments.

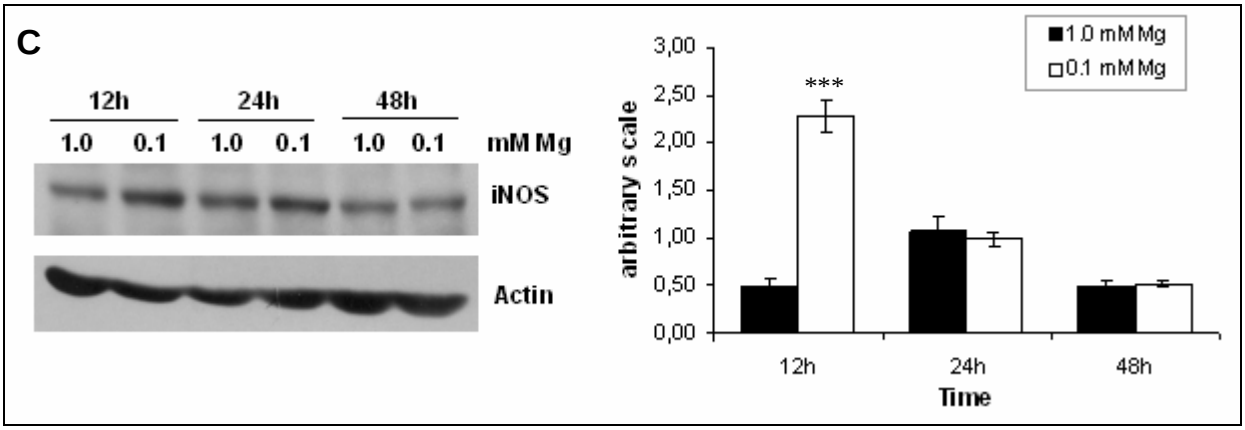
4.8 Mg MODULATES THE RELEASE OF NITRIC OXIDE THROUGH THE UPREGULATION OF iNOS

Because i) NO is crucial for vascular homeostasis and ii) iNOS is a target of NFkB activation, I evaluated whether low Mg affected NOS activity. After 48 hours of culture in media containing either 0.1 or 1.0 mM Mg, NOS activity was higher in HUVEC cells exposed to 0.1 mM Mg than in controls as detected by the Griess method (Figure 4.11A) and by mass spectrometry (Figure 4.11B).

I then evaluated the total amounts of iNOS and eNOS. Since the phosphorylation of Ser^{1177} has been shown to augment enzyme activity, I also determined the levels of eNOS/P-Ser^{1177}. By western blot, an upregulation of iNOS (~ 4 fold) was reproducibly observed in HUVEC cultured in 0.1 mM Mg for 12 h (Figure 4.11C), while eNOS and eNOS/P-Ser^{1177} remained unvaried (Figure 4.11D).







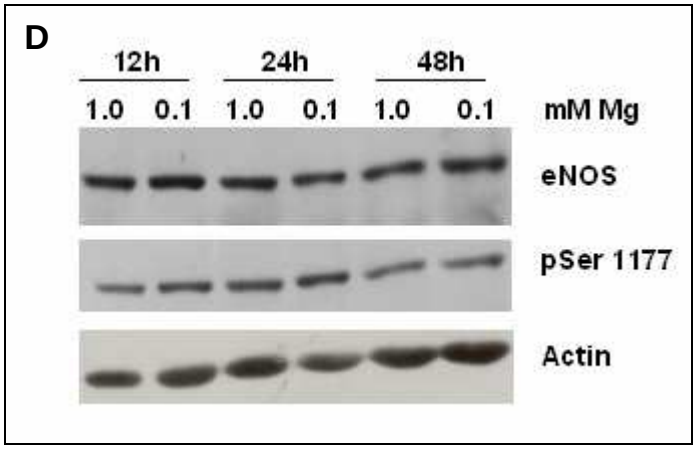


Figure 4.11: NO production and NO synthase amounts in HUVEC in 0.1 or 1.0 mM Mg.

A) - B) NOS activity was measured in HUVEC cultured in medium1.0 mM and 0.1 mM Mg for 48h by using the Griess method for nitrite quantification (A) and GS-MS (GasCromatography mass spectrometry) (B) analysis low histogram. The values were expressed as the mean of five different experiments ± standard deviation (* $p<0.05$).

C) HUVEC were cultured in medium with 1.0 or 0.1mM Mg for different times. Western blot was performed on 80µg of cell extracts using antibodies against iNOS. Actin was used to show that equal amounts of proteins were loaded per lane. The histogram shows the quantitative evaluation of iNOS/actin ratio by densitometry. Significant increases in iNOS total amount are shown as a p value less than 0.01 (*** $p<0.001$). Data are representative of three separate experiments.

D) HUVEC were cultured in medium with 1.0 or 0.1mM Mg for different times. Western blot was performed on 80µg of cell extracts using antibodies against eNOS P-Ser^{1177} , total eNOS. Actin was used to show that equal amounts of proteins were loaded per lane. Data are representative of three separate experiments.

5. RESULTS-2

Mg in microvascular endothelial cells.

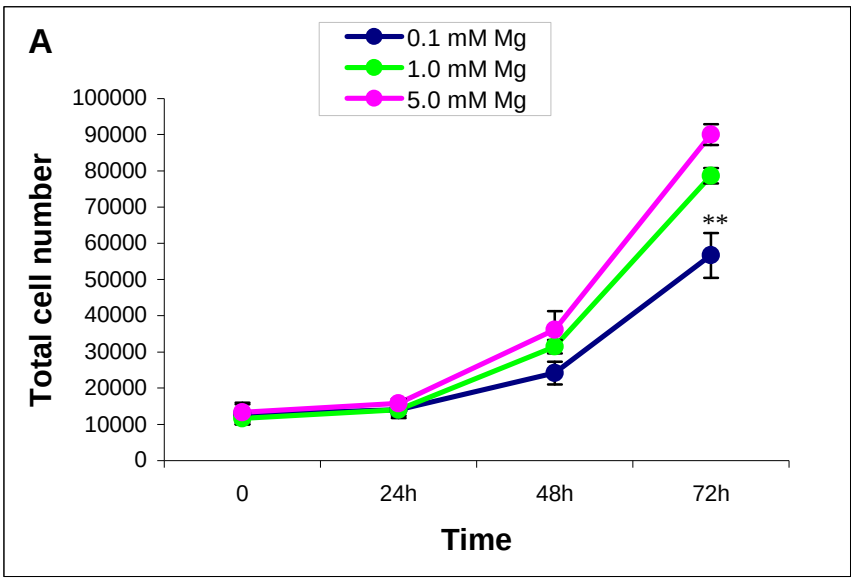
5.1 LOW Mg INHIBITS MEC PROLIFERATION AND MIGRATION

Endothelial cells are highly heterogeneous (Lang I et al., 2003); indeed, morphological and functional differences have been observed in cells isolated from large and small vessels and between cells derived from various microvascular beds. I therefore extended part of my studies to a model of human microvascular endothelial cells (MEC). Since microvascular cells are protagonists in angiogenesis, inflammation and repair, I pointed my attention on events that are critical in the aforementioned conditions, i.e. proliferation, migration, metalloprotease and cytokine synthesis.

Similarly to HUVEC, MEC were cultured in 5.0, 1.0 and 0.1 mM Mg for various times. As expected, low Mg inhibited MEC proliferation (Figure 5.1A). It is relevant to point that this inhibitory effect was reversible upon re-addition of Mg to the culture media (not shown).

Accordingly, when propidium-stained cells were analyzed by flow cytometry, I found that MEC tend to accumulate in the G0/G1 and G2/M phases of the cell cycle, similarly to HUVEC (Figure 5.1B).

Growth inhibition by low Mg correlated with a 3 fold increase of p21 (WAF1) and a 3 fold reduction of Cdc25B (I. Nilsson et al., 2000), as detected by western blot followed by densitometry (Figure 5.1C).



B

Concentration of extracellular Mg [mM]	Distribution (%)			
	Apoptosis	G0/G1	S	G2/M
1.0	11.0 ± 2.2	52.7 ± 4.5	44.3 ± 4.0	2.0 ± 0.3
0.1	11.1 ± 2.3	61.5 ± 4.8	33.3 ± 3.3	5.0 ± 0.6

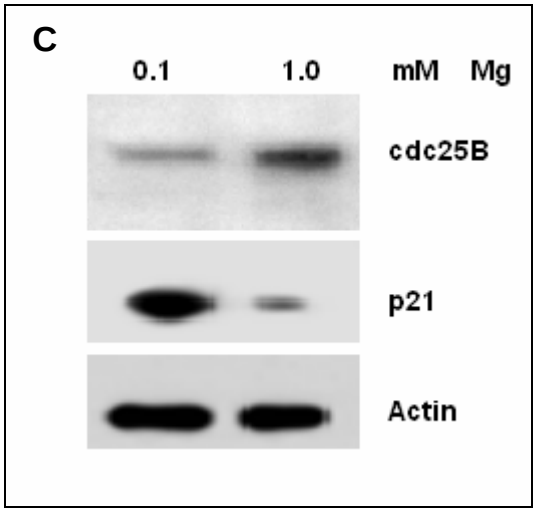


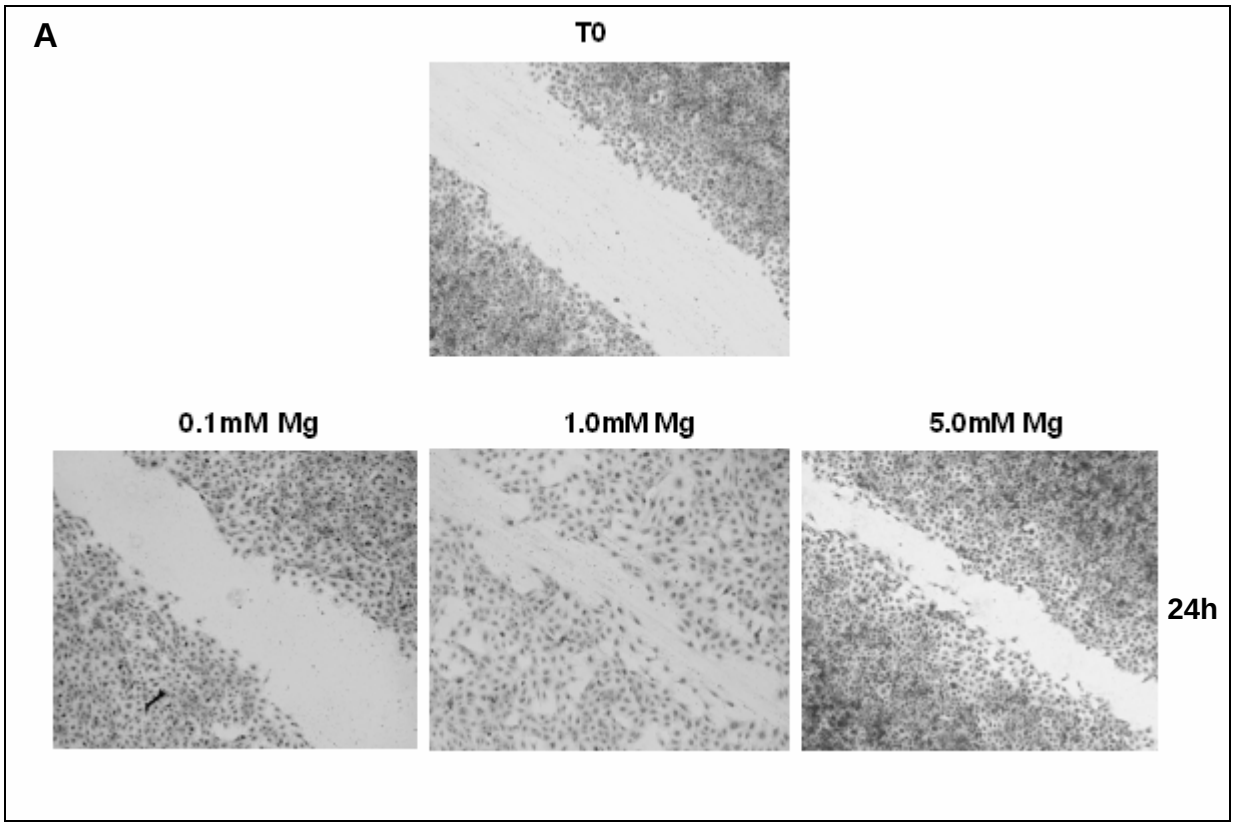
Figure 5.1: Effect of different concentrations of Mg on MEC proliferation.

A) MEC were seeded at low density and cultured in medium with different Mg concentrations. Every 24 hours MEC were trypsinized and viable cells were counted with Burker chamber. Results are expressed as mean ± S.D. of three separate experiments. Significant difference are indicated as a p value less than 0.01 (** $p<0.01$).

B) Cell cycle distribution in MEC in 0.1 or 1.0 mM Mg as detected by flow cytometry on propidium iodide-stained cells. Data are mean ± SD of three separate experiments.

C) Cdc 25B and p21 levels in MEC grown 0.1 or 1.0 mM Mg. Cell extracts from MEC cultured in 0.1 or 1.0 mM Mg for 72 h were subjected to western blot with anti-cdc25B and anti-p21 antibodies. Actin is used as a control of loading.

Mg has been proposed to serve as a chemotactic factor for human endothelial cells (Lapidos KA et al., 2001). Indeed, low extracellular Mg inhibits and high extracellular Mg induces HUVEC migration, partly through the modulation of the tyrosine phosphatase HD-PTP (Baldoli et al., 2010). To assess whether Mg is involved in the regulation of MEC motility, I evaluated the migration of MEC cultured in 0.1, 1.0 and 5.0 mM Mg by wound assay (Figure 5.2A). The wound area was calculated by ImageJ software and expressed using an arbitrary value scale. MEC cultured in 0.1 mM Mg migrate less than the controls in 1.0 mM or 5.0 mM Mg (Figure 5.2B).



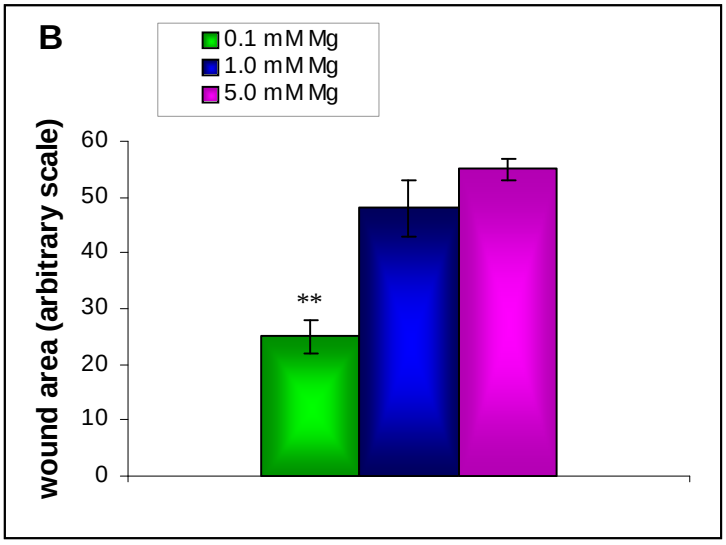


Figure 5.2: Effect of different concentrations of Mg on MEC migration

A) MEC were cultured for 48 h in 0.1, 1.0 or 5.0 mM Mg before wounding. Migration was evaluated 24 h later. Phase contrast microphotographs show the result of a typical assay (10x magnification). T0 indicates the beginning of the experiment, immediately after wounding the monolayer; 24h indicates the end of the experiment.

B) The wound area was calculated by InageJ software and expressed using an arbitrary value scale. Data are shown as the mean ± standard deviation (S.D.) of three different experiments ** $p<0.01$ vs. Control.

5.2 EFFECT OF ANTIOXIDANTS ON MEC PROLIFERATION

Based on the results in HUVEC in low Mg exposed to trolox and apocynin, I anticipated that antioxidants might rescue MEC growth in low Mg. Therefore, MEC in 0.1 or 1.0 mM Mg were cultured in the presence or not of trolox and apocynin. Every 24 h, the cells were trypsinized and counted. Figures 5.3A and B show that Trolox and apocynin do not exert any effect on growth inhibition by low Mg in MEC grown in 0.1 mM Mg.

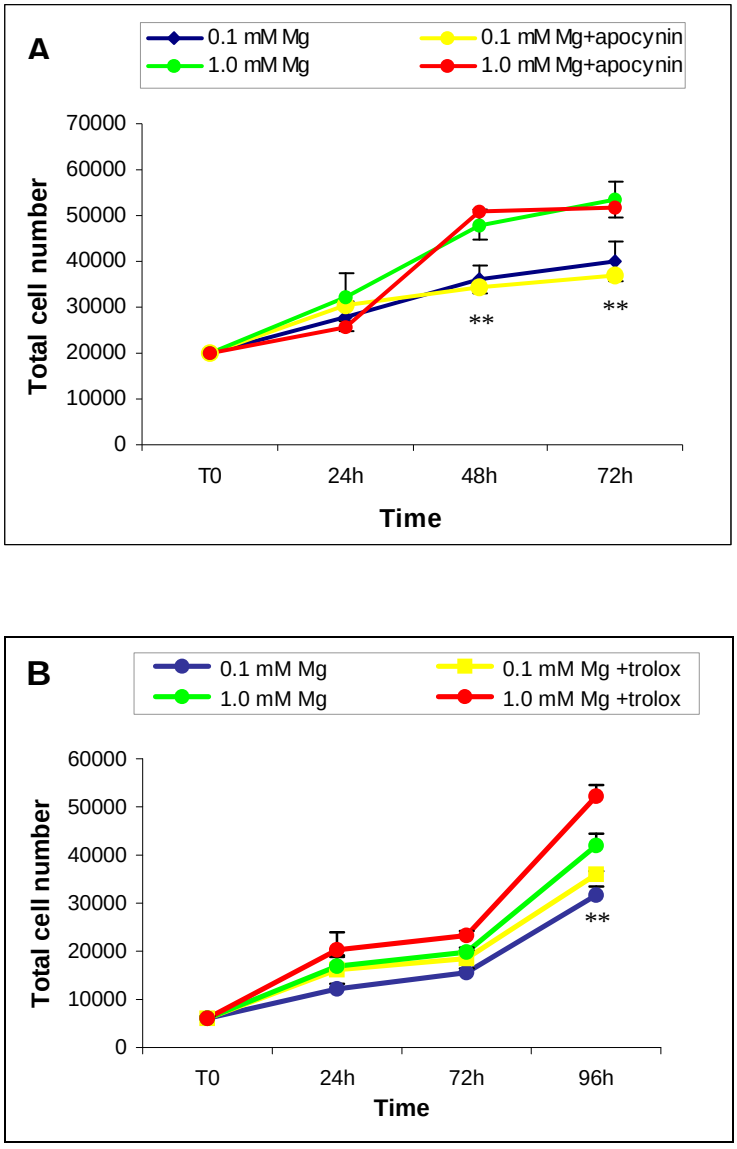
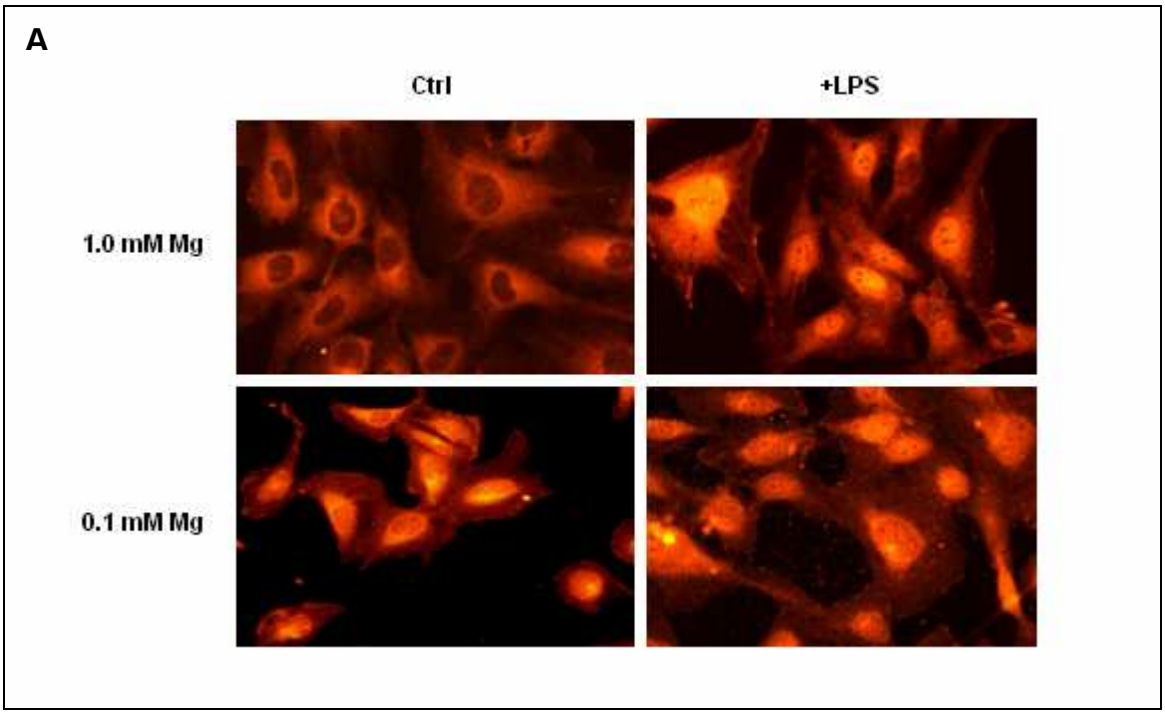


Figure 5.3: The antioxidants trolox and apocynin do not rescue MEC proliferation in low Mg.

MEC were seeded at low density and cultured in medium with 0.1mM Mg concentration and with or without apocynin (10µM) **(A)** or trolox (40µM) **(B).** Every 24 hours, the cells were tripsinized and viable were counted with Burker chamber. Results are expressed as mean ± S.D. of three separate experiments. Significant difference are indicated as a p value less than 0.01 (** $p<0.01$).

5.3 ACTIVATION OF NFkB IN Mg DEFICIENT HD-MEC

Since that low Mg activates NFkB in HUVEC, I performed similar experiments in MEC. To assess the nuclear translocation of NFkB, I performed an immunofluorescence using an antibody anti-p65. Figure 5.4A clearly shows that p65 is nuclear traslocated in MEC in 0.1mM Mg. LPS was used as a positive control. Successively, the functional induction of NFkB was evaluated by reporter gene assays using a vector containing multiple copies of an NFkB response element that drives transcription of the luciferase reporter gene *luc2P* (*Photinus pyralis*). The construct was transiently transfected into MEC and the luciferase activity was measured as described in Matherials and methods. Luciferase activity was induced in cells cultured in 0.1 mM Mg. Again LPS used as a positive control markedly induced NFkB activity (Figure 5.4B).
These results demonstrate that NFkB is transcriptionally active in MEC cultured in 0.1 mM Mg.



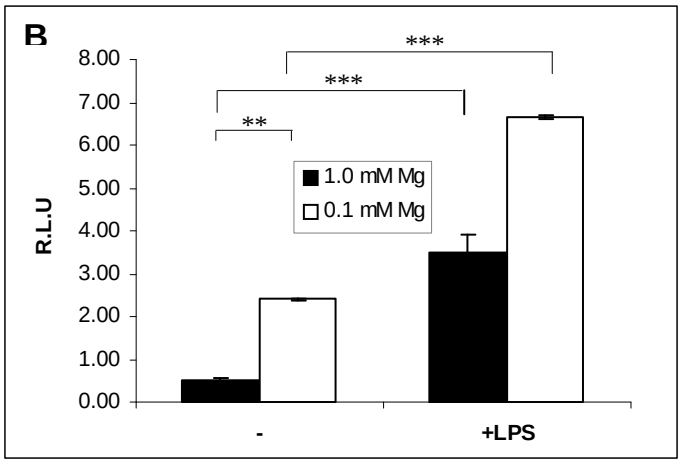


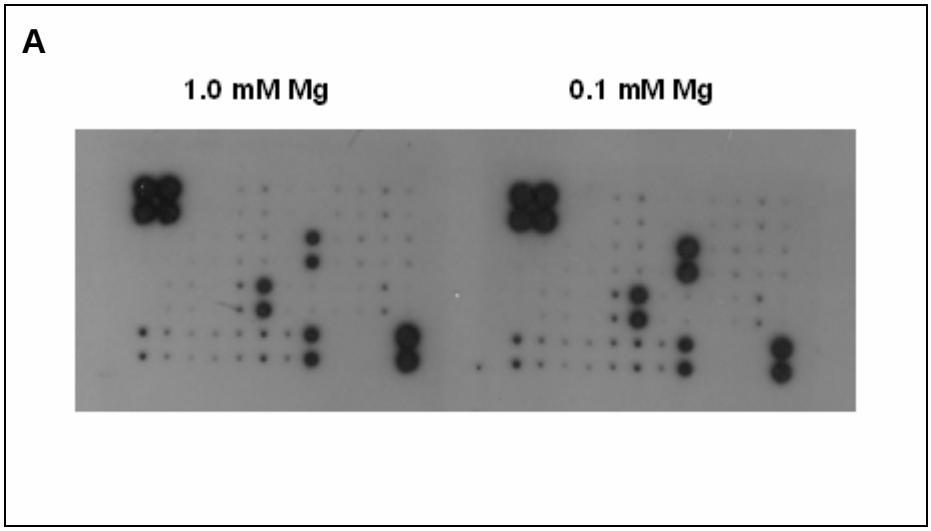
Figure 5.4: NFkB in MEC cells.

A) MEC were cultured on coverslips in medium 0.1 or 1.0 mM Mg for 1h. After the incubation the cells were fixed, stained with antibody against the p65 subunit followed by rhodamine labeled anti-rabbit IgGs and analyzed using a fluorescence microscopy (magnification 40x). **Ctrl**: untreated MEC in 1.0 or 0.1 mM Mg; **+ LPS:** MEC in 1.0 or 0.1 mM Mg treated for 1 h with LPS. Three independent experiments with the same results were performed.

B) Luciferase assay was performed in HUVEC cultured in 0.1 or 1.0 mM Mg as decribed in Materials and methods. Luciferase activity was detected by fluorimetry. Results are shown as the mean ± SD of three separate experiments (** $p<0.01$, *** $p< 0.001$).

5.4 MODULATION OF MOLECULES INVOLVED IN INFLAMMATION AND ANGIOGENESIS IN Mg DEFICIENT MEC

To study whether culture of MEC in low Mg modulates the levels of proteins involved in inflammation and angiogenesis, we utilized a specifically tailored protein array (Figure 5.5A). The results were quantified by densitometry (Figure 5.5B) which shows a significant increase of IL-8 and MCP-1 secretion in MEC cultured in 0.1 mM Mg.



	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	eotaxin	eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	POS	POS	NEG	NEG	eotaxin	eotaxin-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1 α	MIP-1 β	MIP-1 δ
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1 α	MIP-1 β	MIP-1 δ
7	RANTES	TGF-β1	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS
8	RANTES	TGF-β1	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

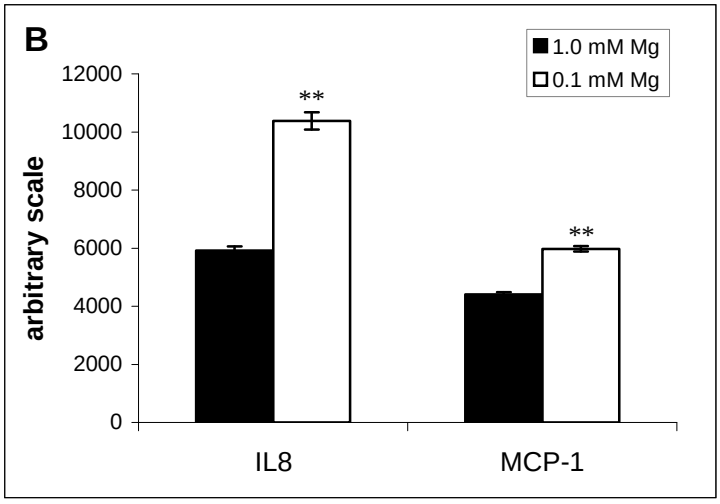


Figure 5.5: Modulation of molecules involved in atherogenesis and inflammation in Mg deficiency MEC.

A) Protein arrays on media collected from human MEC cultured in 0.1 or 1.0mM Mg for 48h. The histogram showsn the densitometric analysis on some spot array and data are expressed as the mean ± S.D. of two different experiments (** $p<0.01$).

B) Densitometric analysis on array was performed and normalized vs controls. Data are expressed as the mean ± S.D. of two different experiments (** $p<0.01$).

5.5 MEC CULTURED LOW Mg DO NOT MODULATE METALLOPROTEASE (MMP) ACTIVITY.

Since MMPs degrade the extracellular matrix, they play an important role in vascular remodeling, cellular migration and angiogenesis. Quiescent endothelial cells produce little or no active MMPs, but these proteases are strongly induced and activated in capillary sprouts during wound healing, inflammation and angiogenesis. Since low Mg stimulates MMPs in HUVEC (see above), I have evaluated the effect of Mg deficiency on MMP-9 (gelatinase A) and MMP-2 (gelatinase B) activity in cells cultured in 0.1 or 1.0 mM Mg by zymography and found no significant differences (Figure 5.6). This result highlights a different response to low Mg of MEC vs HUVEC.

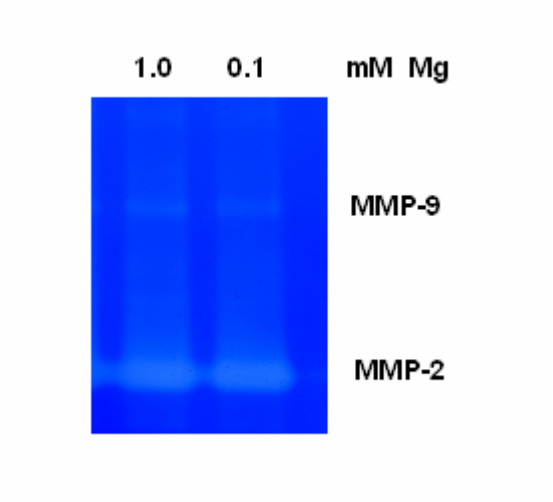


Figure 5.6: Effect of low Mg on MMP secretion.

Representative zymography were performed on culture media from cells cultured in 0.1 or 1.0mM Mg as described in Materials and Methods.

6. RESULTS-3

TRPM7 in micro- and macrovascular endothelial cells.

6.1 MODULATION OF TRPM7 EXPRESSION IN ENDOTHELIAL CELLS

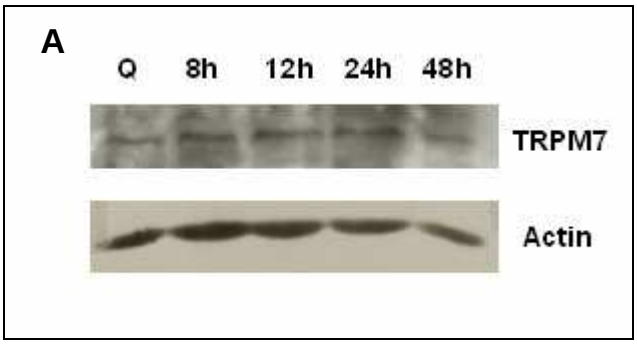
Although Mg is an abundant cytosolic cation important in many biological processes, little is known about the transport mechanisms that regulate its homeostasis, especially in vascular cells. Transporters and exchangers that have been implicated include Na/Mg exchanger, Mg/Ca exchanger and more recently, two novel ion channels of tha melastatin-related transient receptor potential subfamily TRPM6 and TRPM7 were identified as critical regulators of vertebrate intracellular Mg levels in different cell types. Recently, TRPM7 has been shown to be essential for Mg homeostasis in mammals (Ryazanova et al., 2010). The presence of functional TRPM7 channels in human endothelial cells has also been shown (Inoue et al., 2006). In addition, the inhibition of TRPM7 has been reported to promote endothelial cell growth and nitric oxide production (Koichi Inoue and Zhi-Gang Xiong, 2009). Since TRPM7 is implicated in Mg homeostasis and Mg affects endothelial proliferation and function, I examined the modulation of the expression of TRPM7 in human macro- and microvascular cells and how its silencing impacts on some endothelial functions.

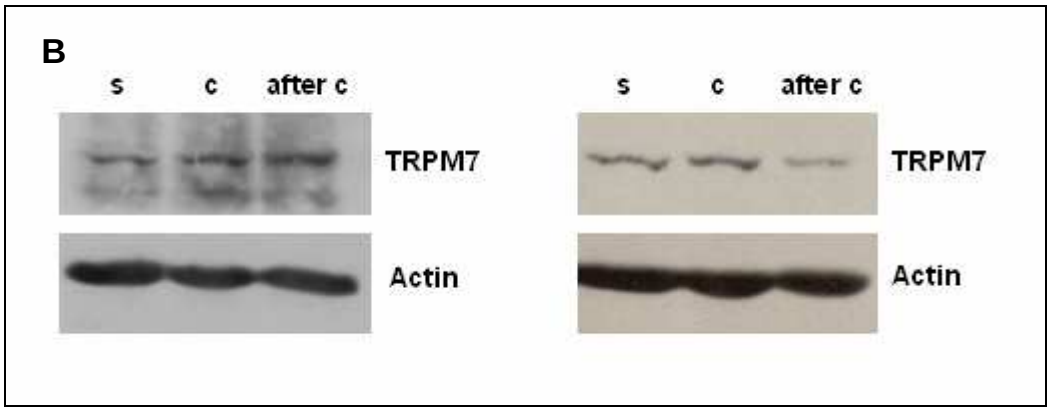
To evaluate whether the expression of TRPM7 was related to cell proliferation, HUVEC were growth arrested by exposure to a starvation medium. Two days later, the cells were exposed to the complete growth medium for various times to stimulate their entry in the cell cycle. Under these conditions, the entry into the S phase occurs after 24 h from the re-addition of growth medium as detected by ^{3}H-Thymidine incorporation assay (not shown and Bolognese et al, 2006). Upon re-entry in the cell cycle, no differences of the total amounts of TRPM7 were detected (Figure 6.1A).

To further explore the relation between the levels of TRPM7 and proliferation/quiescence, I compared proliferating vs quiescent HUVEC and MEC for their amounts of TRPM7. To this purpose I compared the levels of TRPM7 in sparse, confluent and post-confluent cells, and found no significant differences in the total amounts of TRPM7 (Figure 6.1B).

These results indicate that the amounts of TRPM7 are not dependent upon quiescence, either induced by contact inhibition or by starvation.

Because i) low Mg promotes inflammation and ii) inflammatory cytokines inhibit endothelial growth, I asked whether the prototypical inflammatory cytokines IL-1 and TNF-α modulated TRPM7. As shown in figure 6.1 C, both IL-1 and TNF-α induce TRPM7 in HUVEC and MEC.





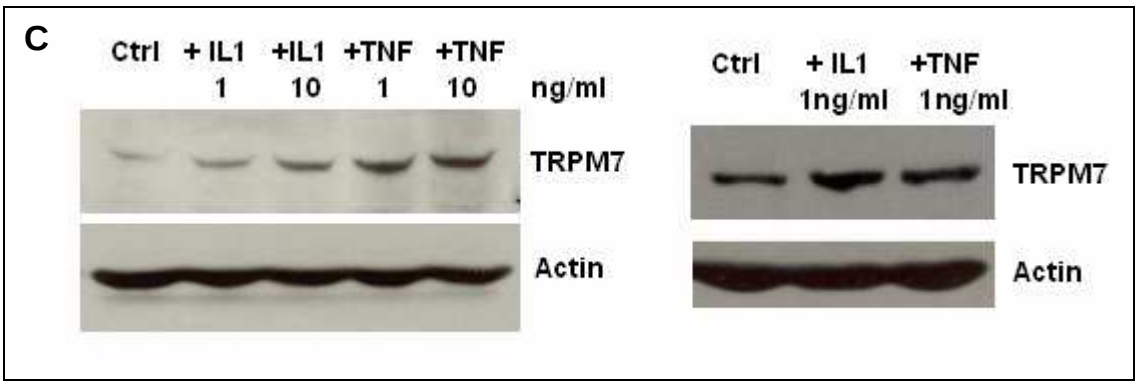


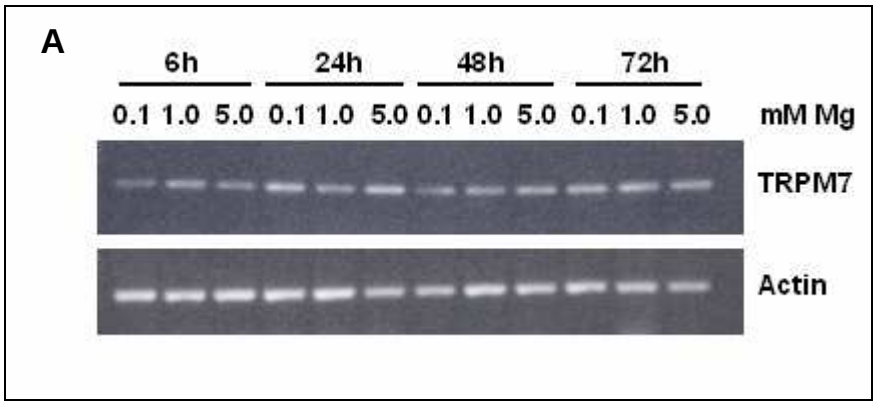
Figure 6.1: TRPM7 amounts in quiescent, proliferating endothelial cell.

A) Subconfluent HUVEC were starved as described for 48h. Then cells were exposed for different times to complete growth medium to induce the re-entry in the cell cycle (Q: quiescent cells). After the hours of incubation, the cells were lyzed and cells extracts were utilized for western blot using anti-TRPM7 antibodies. Actin was used to show that equal amounts of proteins were loaded for lane.

B) HUVEC (left) and MEC (right) were lyzed 2 days before reaching confluence (s: sparse), the day they reached confluence (c: confluent) and 2 days after reaching confluence (after c). Western blots with anti-TRPM7 and anti-actin antibodies were performed.

C) HUVEC (left) were exposed to different concentrations of the inflammatory cytokines IL-1 and TNF-α (1 and 10 ng/ml) for 24h. MEC (right panel) were treated with IL-1 and TNF (10 ng/ml) for 24 h. A representative western blot with anti-TRPM7 and anti-actin antibodies is shown.

As shown above, low extracellular Mg retards, while high Mg promotes endothelial growth-proliferation. I therefore investigated whether TRPM7 was modulates by different extracellular concentrations of Mg ion. HUVEC and MEC were cultured in different concentrations of extracellular Mg for 3 days. In HUVEC, while the TRPM7 expression remained mainly the same as detected by semiquantitative RT-PCR (Figure 6.2A), by western blot I observed that the total amounts of TRPM7 were increased by approximately 2 fold in 0.1 mM Mg and decreased of about 3 fold in 5.0 mM Mg. On the contrary, in MEC TRPM7 total amounts are decreased in cells cultured in low Mg when compared to controls while they remain unvaried in cells cultured in 5.0 mM Mg vs controls (Figure 6.2B).



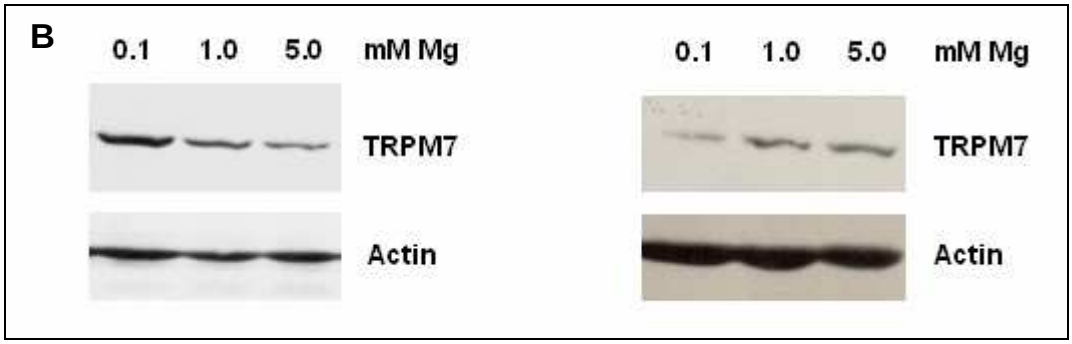


Figure 6.2: TRPM7 expression in endothelial cells cultured in different Mg concentration.

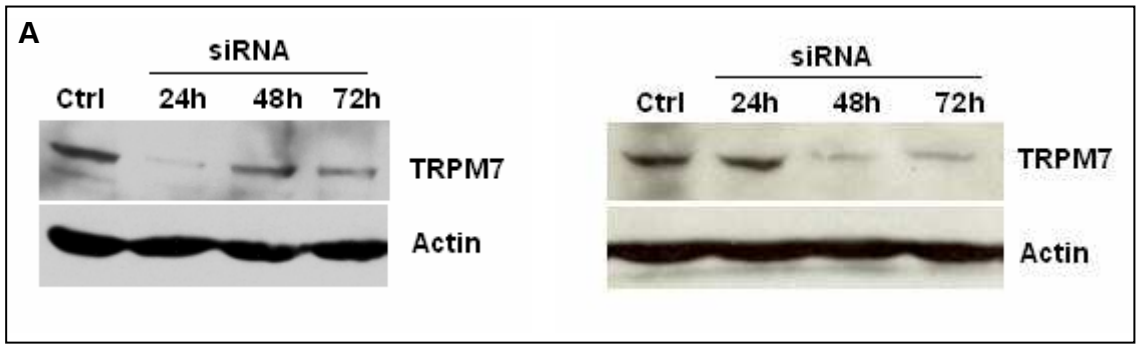
A) Semiquantitative RT-PCR was performed on RNA extracted from HUVEC cultured in different concentrations of Mg using primers designed on TRPM7 sequences (see Materials and Methods). Actin was used as a control of loading.

B) HUVEC (left) and MEC (right) were cultured in different concentrations of Mg. After 3 days, the cells were lyzed and cell extracts were utilized for western blot using anti-TRPM7 antibodies (Abcam). Actin was used as a control of loading.

6.2 EFFECT OF TRPM7 SILENCING ON THE PROLIFERATION OF ENDOTHELIAL CELLS

TRPM7 has been reported to affect the growth/proliferation of various cell types (Jiang J et al., 2007; Schmitz C et al., 2003; Wei WL et al. 2007 and Gwanyanya A et al., 2006). To determine the importance of TRPM7 in the proliferation of endothelial cells, TRPM7 was downregulated by siRNA. I transiently transfected sub-confluent HUVEC and MEC cells with specific, commercial siRNA using HiPerfect Transfection Reagent (Quiagen). As a control, a non-silencing siRNA sequence was used. After 4h incubation, an equal volume of complete medium was added to the transfection medium. After 24h, 48h and 72h from transfection the cells were washed with PBS, scraped and lysed. The total amounts of TRPM7 were examined with western blot. TRPM7 levels were dramatically reduced 24h after transfection and began to increase after 48h in HUVEC, while in MEC the expression was markedly reduced after 48h and 72h from transfection (Figure 6.3A).

In parallel, I silenced both HUVEC and MEC with siRNA anti-TRPM7 and a non-silencing sequence. Every 24h I counted the viable cells up to 72h. Figure 6.3B shows a significant induction of cell growth in the HUVEC silencing TRPM7 vs their controls. On the contrary, MEC silencing TRPM7 proliferate slower than controls.



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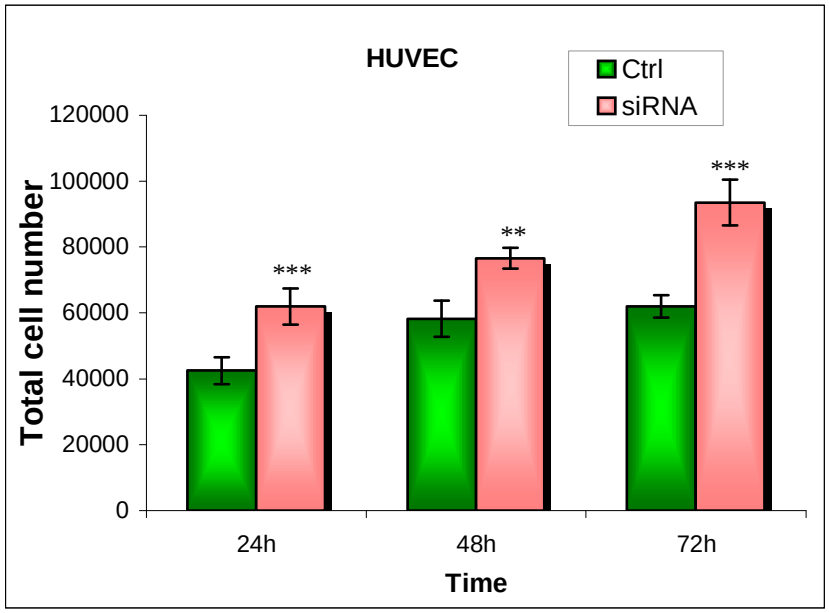

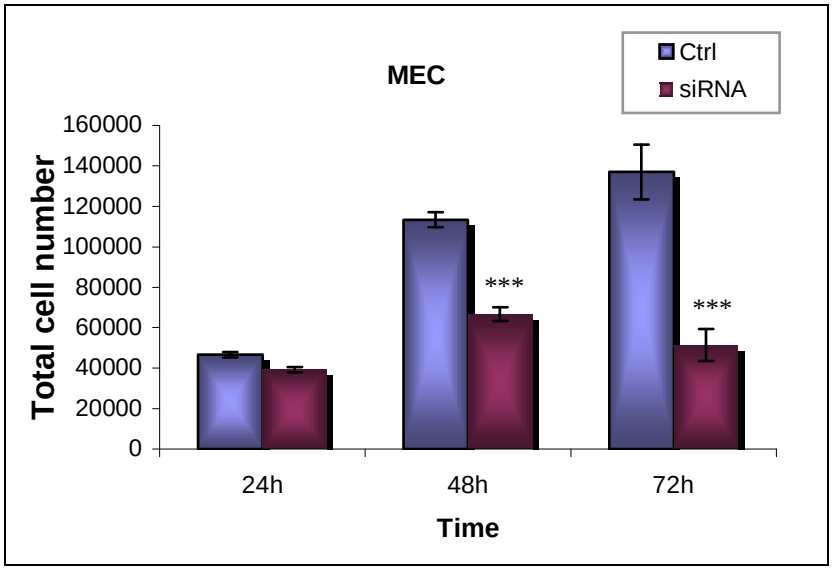

Figure 6.3: Silencing of TRPM7 in HUVEC and MEC cells.

A) HUVEC (left panel) and MEC (right panel) were transiently transfected with or without a commercial siRNA against TRPM7 for 24h, 48h and 72h.Western blot analysis was performed with an antibody anti-TRPM7 on cell extracts of siRNA transfected and their controls. Actin was used to show that equal amounts of proteins were loaded per lane.

B) HUVEC (up panel) and MEC (down panel) were transiently transfected with a siRNA against TRPM7 or a non-silencing sequence. At the indicated time intervals, the cells were harvasted by trypsin and viable cells were counted. Data refer to means ± SD of three separate experiments performed in triplicate. Significant variations are indicated as a p value less than 0.01 (** $p<0.01$) or than 0.001 (*** $p<0.001$).

6.3 EFFECT OF TRPM7 SILENCING ON eNOS EXPRESSION AND NO PRODUCTION.

To determine whether changes in TRPM7 expression also affect the level of eNOS, eNOS expression was examined after silencing TRPM7. As shown in Figure 6.4A the total amounts of eNOS were not affected TRPM7-siRNA transfection of HUVEC. NO was then measured in TRPM7-siRNA transfected HUVEC and their controls. A slight, albeit reproducible, induction of NO realease was detected (Figure 6.4B). The basis of this increase is under investigation at the moment.

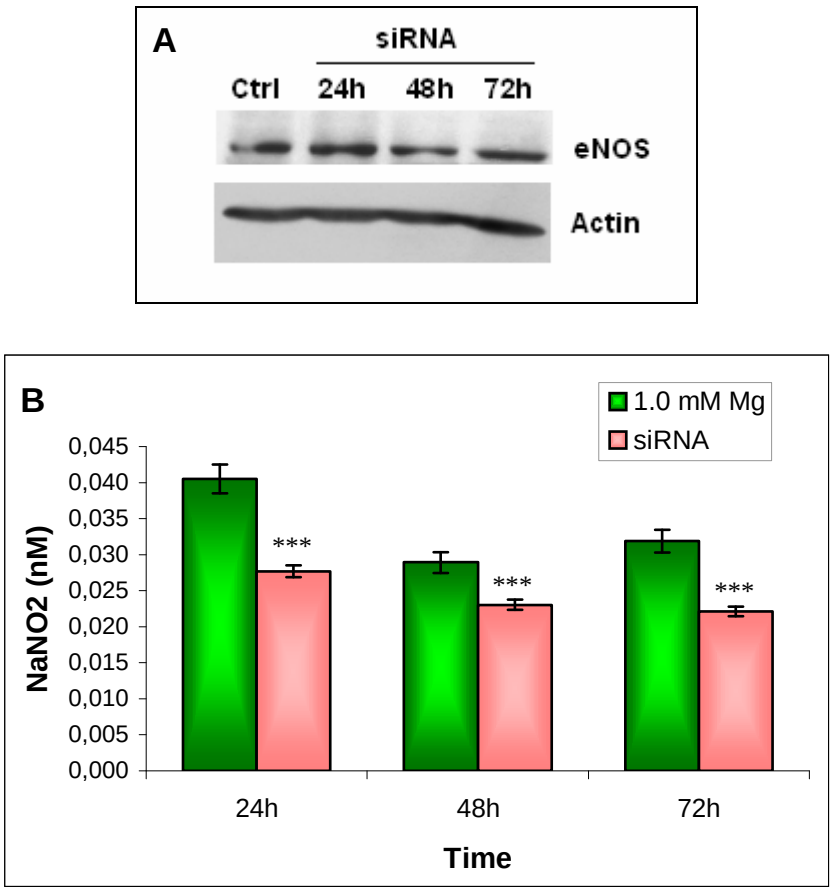

Figure 6.4 : Effect of TRPM7 on eNOS total amounts and NO release in HUVEC.

HUVEC were transiently transfected with a siRNA against TRPM7 for 24, 48 and 72 h.

A) Western blot was performed on 80 µg of cell extracts using antibodies against eNOS. Actin was used to show that equal amounts of proteins were loaded per lane.

B) NOS activity was measured by using the Griess method for nitrate quantification. The values were expressed as the mean of 5 different experiments ± standard. Significant variations are indicated as a p value less than 0.001 (*** $p<0.001$).

6.4 EFFECT OF TRPM7 SILENCING ON THE MIGRATION OF ENDOTHELIAL CELLS

TRPM7 also modulates the migration of various cell types (Callera GE et al., 2009; Elie Abed and Robert Moreau, 2009). Endothelial migration is an early step in angiogenesis, which is crucial in responding to tissue demands in physiological and pathological conditions (Lamalice L et al., 2007). Migration is also important after injuries to the vessel wall, i.e. after mechanical stress such as shear stress or cateterization, as an attempt to heal the damage (Vanhoutte PM. 2009). Mg is known to modulate endothelial migration (Lapidos et al., 2001; Baldoli et al., 2010). I therefore investigated whether TRPM7 is involved in regulating the migration of endothelial cells by transiently silencing TRPM7. Wound assay showed that serum-stimulated migration of MEC was inhibited by TRPM7 silencing, whereas silencing TRPM7 in HUVEC induced cell migration (Figure 6.5).

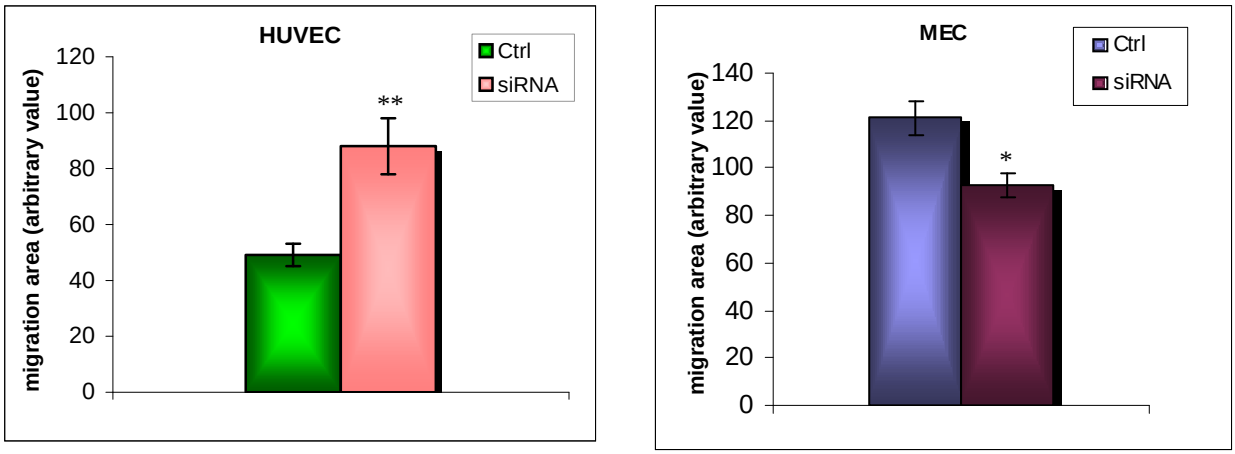

Figure 6.5: Effect of TRPM7 silencing on cell migrationin MEC and in HUVEC.

HUVEC and MEC were silenced and then cultured for 24 and 48h, respectively. Migration was evaluated 24 h later. The wound area was calculated by InageJ software and expressed using an arbitrary value scale. Data are shown as the mean ± standard deviation (S.D.) of three different experiments; *p<0.05 and ** p<0.01 vs. control.

7. DISCUSSION

Magnesium (Mg) is a versatile electrolyte shown to be involved in many cellular processes. It functions as a cofactor in the energy metabolism, nucleotide and protein synthesis and as a regulator of sodium, potassium and calcium channels. Several hundreds of enzymes, directly or indirectly (via the need of Mg-ATP), are dependent on Mg. Current dietary guidelines recommend adequate intake of Mg (310-420 mg daily for adult males and females respectively) in order to maintain health and lower the risk of cardiovascular disease. Mg is a cation that mostly resides inside cells of muscles and bones. Only a small amounts, about 1% can be found extracellulary. Serum Mg, the most commonly used parameter to measure Mg levels, correlate with intracellular free Mg levels (Maier JAM. et al., 2004). Hypomagnesemia, a serum level less than 1.5 mEq/l is associated with malabsorption, alcoholism, malnutrition, renal disease and the use of diuretics (McCance & Huerther 2006). Recent evidence from animal models and clinical studies on humans suggests that low serum concentrations of Mg are associated with inflammation, atherogenesis and endothelial dysfunction (Nielsen FH 2010; Mazur et al., 2007; Maier JAM et al., 2004; Shechter M et al., 2000). Indeed Mg plays an important role in the etiology of diabetes, myocardial infarction, hypertension, arrhythmias and congestive heart failure in humans. Mg supplementation can significantly decrease blood pressure and stabilize cardiac arrhythmias and acute myocardial infarction (Gazmuri RJ et al., 2001; Singh J et al., 1997; Shechler M 2010).

Endothelial cells (EC) are implicated in atherogenesis, thrombosis, and angiogenesis (Cined DB et al., 1998). Far from being homogeneous, the endothelium represents a consortium of groups of cell located within blood vessels of different tissue (Lang et al., 2003). Several factors influence this heterogeneity including: i) morphological and functional differences between large and small vessels and between cells derived from various microvascular endothelial beds; ii) different response to growth factors; iii) organ specificity reflecting the cumulative expression of unique genes under the control of organ-specific regulatory elements; iv) pathological conditions, such as tumor growth, which is accompained by the development of a characteristic tumor vasculature. The diversity of the vascular bed is crucially determined by the type of endothelial cells lining the inner vessel surface. HUVEC and MEC are the most common cell culture systems for analyzing human macro- and microvascular endothelial cells *in vitro*. Clear evidence has been provided for differences in basal gene expression profiles of HUVEC and MEC (Chi JT et al., 2003). Recently, the relevance of the diversity of HUVEC and MEC for the response to inflammatory stimuli has been defined (Dorothee Viemann et al.,

2006). Using oligonucleotide microarray technique, the TNF-induced expression profile in MEC and HUVEC has been systematically analyzed on 13000 human genes to reveal that about half of the TNF-induced genes were specific for MEC or HUVEC. Interestingly, the majority of those genes regulated depending on the cell type encoded for chemokines, cytokines, and cell surface molecules.

Being low magnesium involved in promoting inflammation, with my work I strive for a comparison of the modulation of *in vitro* behaviour of MEC and HUVEC to low Mg.
In this study, I addressed two main issues: i) the modulation of endothelial function by low extracellular Mg and ii) the expression and the role of the Mg transporter TRPM7 in endothelial cells. Because endothelial cells are highly heterogeneous, I utilized macro and microvascular endothelial cells.

7.1 Mg in macrovascular endothelial cells.

Since Mg deficiency promotes atherosclerosis, thrombosis and hypertension, I asked whether low concentrations of Mg could affect endothelial behavior. I began by focusing on the effect of low Mg on HUVEC proliferation. As most cell constituents are doubled in their amount during G1 phase of the cell cycle, it is not surprising that the reduction of extracellular Mg inhibits cell proliferation. However, the role of Mg in modulating eukaryotic cell growth is controversial. Whereas studies in normal cells have established a positive relation between the levels of Mg and the extent of proliferation, conflicting results have been obtained in tumor cells. For instance, low Mg affected the growth of 3T3 fibroblasts, but not of their transformed counterpart (Rubin H, 2005). In accordance, it is noteworthy that Mg-deficiency delays the transit through the G1 and S phase of normal but not transformed cells (Littlefield NA et al., 1991). I found that low extracellular Mg reversibly inhibits HUVEC growth by increasing the number of cells in the G0/G1 and G2/M phases and decreasing the number of cells in the S phase of the cell cycle. Growth retardation is rescued by Mg supplementation, thus suggesting that no permanent modifications occur as a consequence of the deprivation of Mg, and it is independent from the salt used. In agreement with the results obtained by flow citometry, I found that growth inhibition correlates with the upregulation of the cdk inhibitor p21 and the marked downregulation of the levels of Cdc25B, a phosphatase which plays a key role in controlling G2-M progression (Nilsson et al., 2000). The upregulation of p21 might be responsible for the accumulation in the G1 phase of HUVEC cultured in low Mg, while the reduction of the levels of Cdc25B might explain the negative regulation of the G2/M transition, as previously demonstrated in HUVEC treated with the phorbol ester PMA (Kosaka C et al., 1996).

Considering the association of Mg deficiency with oxidative stress (Guerrero-Romero F and Rodrìguez-Moràn, 2006), it is noteworthy that free radicals inhibit endothelial growth. Interestingly, it has also been shown that Mg deficiency makes cells more sensitive to oxidative stress by impairing to their scavenger systems, including GSH (Zhou Q et al., 1999). Free radicals can damage DNA, dramatic event that can induce apoptotic cell death or irreversible proliferative arrest, as occurs in cellular senescence (Zhou Q et al., 1999) which is also accelerated by Mg deficiency (Ferrè S et al., 2007).

Exposing HUVEC to low Mg, I observed no alterations of DCF-detectable intracellular ROS under basal conditions. Interestingly, early after exposure to 0.1 mM Mg, HUVEC were more sensitive to the oxidant action of H_2O_2 than the cells cultured in 1.0 mM Mg. Indeed, in the presence of H_2O_2, I observed an increase of DCF-detectable ROS in Mg-deprived HUVEC

compared to controls. Similar results were obtained in myocardium (Manju L et al., 2006). Probably the addition of H_2O_2 unmasks a border-line situation characterized by mild oxidative stress induced by low Mg, which, however, does not overwhelm the anti-oxidant capabilities of the cells. Indeed, I can not exclude that a modest increase of ROS after Mg deficiency might escape DCF detection due to the time and the duration of this phenomenon. It is noteworthy that antioxidants such as apocynin and trolox prevented low Mg-dependent inhibition of HUVEC proliferation, thus indicating a role of oxidative stress in modulating endothelial growth retardation in low Mg.

In atherogenesis, oxidative stress mediates signalling pathways underlying vascular inflammation, starting from the initiation of fatty streak development, through lesion progression, to ultimate plaque rupture (Monaco C. et al., 2004). Being endothelial dysfunction a critical step in atherogenesis, it is noteworthy that low Mg induces interleukin-1α (IL-1α), VCAM and PAI-1 and promotes a senescent phenotype in cultured endothelial cells (Maier JAM. et al. 2004; Killilea D. et al., 2008). However, how these events are initiated has not been fully elucidated. One possible contributor to the infiammatory response due to Mg deficiency could be the activation of transcription factors NFkB. The NFkB family consists of a group of inducibile transcription factors implicated in the regulation of crucials steps of immune and inflammatory responses through regulation of the gene expression of a large number of cytokines and other immune genes. NFkB is a crucial regulator of inflammation which is activated in experimental and human atherosclerosis (Kempe S. et al., 2005; Denk A. et al. 2000; Monaco C. et al., 2004). I decided to study the role of the transcription factor NFkB in Mg deficient HUVEC. In Mg deficient HUVEC NFkB is rapidly activated. In particular, I have analyzed the prototypical NFkB complex demonstrating the involvement of p50 and p65 in low Mg dependent NFkB activation. My results are in agreement with data obtained in primary cultured cerebral vascular smooth muscle cells demonstrating by EMSA that low extracellular Mg activates NFkB (Altura B.M et al., 2003). In addition, Mg supplementation markedly attenuates NFkB nuclear translocation and protects IkB from degradation in LPS-treated endothelial cells (Altura B.M et al., 2003). Interestingly, in a model of cyclosporine-induced nephrotoxicity in rats, Mg supplementation showed marked beneficial effects by preventing NFkB activation which is responsible for renal damage (Asai T. et al, 2003). However, all these studies are lacking the demonstration of the functional induction of NFkB. Anyway, Mg levels seem to participate to the complex regulation of NFkB activity in various systems. In HUVEC, since Mg deficiency induces oxidative stress, I hypothesize that it is oxidative stress to activate NFkB. Indeed in Mg deficient HUVEC, the anti-oxidant trolox, a synthetic cell-permeable analogue of α-tocopherol,

prevents NFkB activation. I therefore propose that in Mg deficient HUVEC oxidative stress might activate NFkB and trigger NFkB signalling pathway. Accordingly, in HUVEC grown in 0.1 mM Mg the upregulation of IL-1α mediates some of low Mg effects on these cells (Ferrè S et al. 2007). In this study I utilized protein array to obtain a broad profile of the cytokines and growth factors released by HUVEC cultured in low Mg. 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 I show the induction of RANTES, IL-8 and PDGF-BB and I confirm the upregulation of IL-1, all targets of NFkB (Monaco C. et al., 2004). 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 (Ross R. et al, 1993). 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 (Ross R. et al, 1993; Barrett T.B et al., 1987).

The protein array also shows 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. In particular, the enhanced expression of IL-8 and RANTES within human atherosclerotic lesions has been reported (Monaco C. et al., 2004; Zernecke A et al., 2008). IL-8 is a chemokine initially characterized for its leukocyte chemotactic activity but now known to possess pro-angiogenic, pro-inflammatory and pro-atherogenic properties. IL-8 is critical for chemotaxis and adhesion of monocytes to the endothelial cells, a pivotal step in the initiation of atherogenesis. It also promotes vascular smooth muscle cell proliferation and migration, crucial events for the progression of the plaque (Zernecke A et al., 2008) and might be involved in promoting intraplaque angiogenesis.

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 (Zernecke A et al., 2008). I therefore conclude that low Mg stimulates endothelial secretion of IL-8, RANTES and PDGF-BB, all pathophysiological contributors to atherosclerosis (Zernecke A et al., 2008). In our experimental conditions, however, we did not observe any modulation of TNFα and IL-6 which have been shown increased in animal model under a Mg-restricted diet (Mazur A, 2007). We hypothesize that different cell types, other than endothelial cells, might contribute to secrete these cytokines

in vivo.

Protein array also revealed the increase of GM-CSF, a cytokine which is transcriptionally dependent upon NFkB activation. This finding is particularly interesting in the light of a very recent study showing that GM-CSF regulates intimal cell proliferation in the early step of atherosclerosis. More studies are necessary to confirm the increase of GM-CSF in HUVEC cultured in low Mg.

Reactive oxygen species (ROS) are known to react with thiol groups, such as those involved in preserving MMP latency, so they could modulate the activity of MMPs. Moreover the process of vascular remodeling implicated in atherosclerotic plaques involves the enzymatic activity of metalloproteinases (MMPs) which degrade collagen fibrils leading to the loss of fibrous cap integrity. The activation of MMPs by ROS appears to be a key event in arterial remodeling and several studies provide evidence for a link between ROS and MMPs. In cultured smooth muscle cells exposed to mechanical stress, ROS, derived from NAD(P)H oxidase, increase the expression and release of MMP-2 (Karsten G et al., 2003). Also MMP-9 is induced by ROS in IL-1-β-stimulated smooth muscle cells (Gurjar MV et al., 2001). Regulation of MMPs may occurs at multiple levels: i) either by gene transcription and synthesis of inactive proenzyme or ii) post translational 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. I found that Mg deficient HUVEC secreted increased amounts of MMP-2 and -9 and of their inhibitor TIMP-2. 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. Therefore, it is possible 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 (Galis Z.S et al., 1994). Recently, genetic variation within the MMP-2 promoter region was associated with cap thickness and therefore may influence the role of MMP-2 in plaque vulnerability (Volcik K.A. et.al., 2010).

I also analyzed another target gene of activeted NFkB: the inducible form of NOS (iNOS). I found that HUVEC cultured in low Mg produce more NO through the induction of iNOS, while eNOS is not modulated. Once induced, iNOS can synthesize NO at high rates over long periods of time without further stimulus. When NO production is high, it can react with superoxide anion to produce peroxynitrite, which causes lipid oxidation, deamination, alterations in DNA, protein nitration and oxidation, inhibition of iron-centered enzymes involved in mitochondrial

respiration, and inhibition of aconitase activity, all of which can lead to profound cellular disturbances as well as the development of atherosclerotic lesions (Szabo C., 1996). More studies are ongoing on this topic.

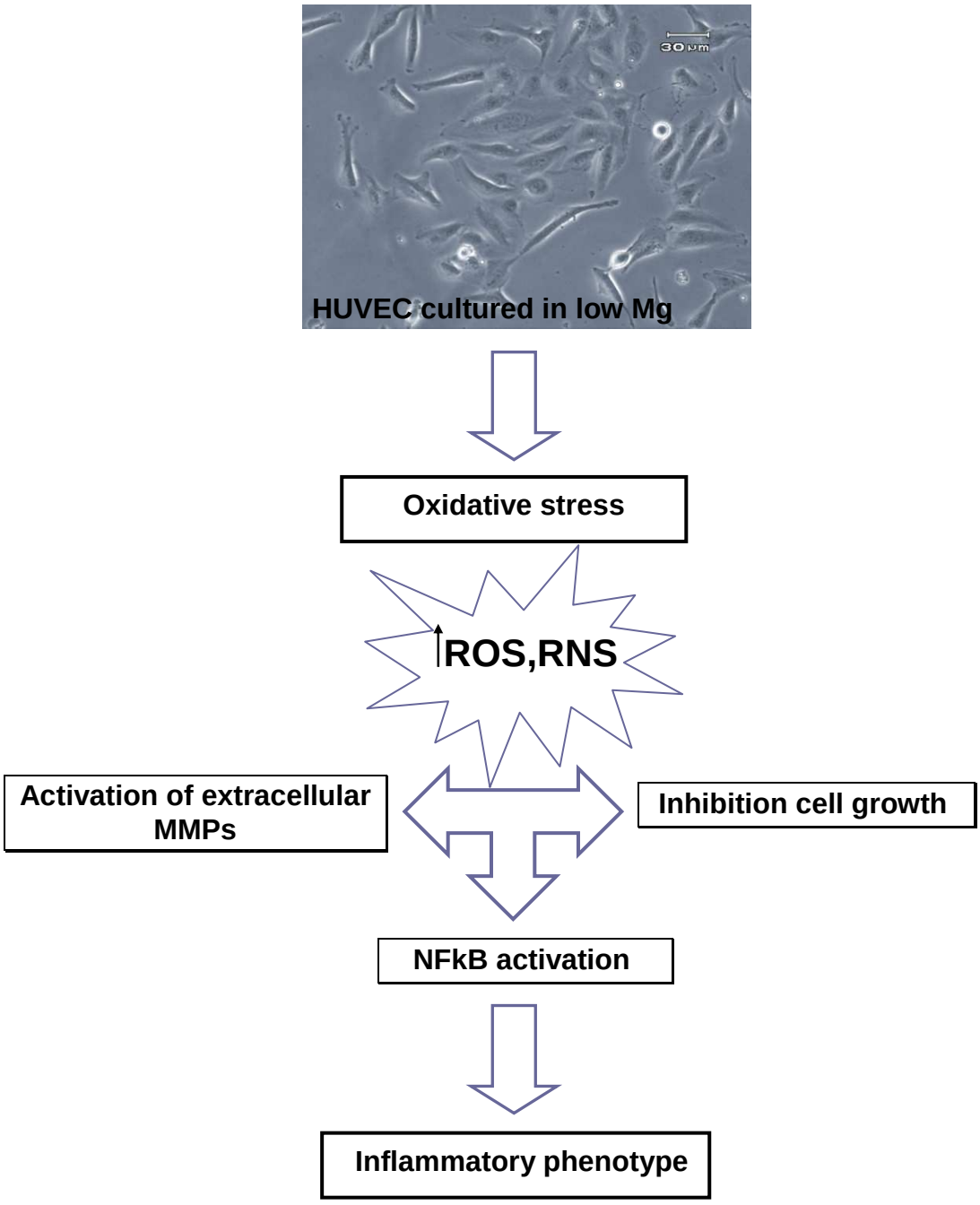

Figure 7.1: Summary of the main findings on HUVEC cultured in low Mg.

7.2 Mg in microvascular endothelial cells

Microvascular endothelial cells are protagonists in angiogenesis and inflammation. Since i) proliferation, protease production and migration are crucial steps in angiogenesis and ii) cytokines have a role both in the formation of new vessels and in inflammation, I evaluated these parameters in MEC.

I found that low Mg inhibits MEC growth and this correlates with the upregulation of p21 and the downregulation of Cdc25B. Accordingly, like in HUVEC, cell cycle analysis demonstrated that low Mg promotes the accumulation of MEC in G0/G1 and G2/M phases of the cell cycle. Differently from HUVEC, however, the inhibition of MEC proliferation is not reverted when the cells are cultured in low Mg and exposed to antioxidants. Growth inhibition by low Mg can be also explained as the result of a Mg deficiency-induced metabolic adaptation, involving the decrease of high energy phosphates and the consequent decline in protein and carbohydrate synthesis. In addition, since Mg is a natural calcium antagonist, it is conceivable that also an imbalance between Ca and Mg may play a role in modulating cell growth. These metabolic events may also play a role in the inhibition of endothelial migration observed in MEC cultured in low Mg. Since Mg is a cofactor of adenylate cyclase and cAMP-dependent phosphodiesterase, I speculate that the reduced migration in low Mg might be dependent on the fact that different Mg levels could alter cAMP, which is implicated in endothelial migration (Hackett SF et al.,1987). Moreover, it is noteworthy that Mg is required for the assembly of actin polymers and for myosin ATPase activity, two key components of the motor responsible for cell migration. Differently from HUVEC, low Mg does not alter MMPs activity in MEC. The inhibition of cell growth and migration might account for the inhibition of tumor angiogenesis observed in mice fed with a Mg-deficient diet (Maier JAM et al., 2007). Cytokines are fundamental both in angiogenesis and inflammation. For this reason, I studied the cytokine network also in MEC cultured in 0.1 vs 1.0 mM Mg. I found that, low Mg importantly stimulates IL8 and MCP-1 release in low Mg MEC. Moreover I found that low Mg actives NFkB also in MEC. I conclude that low Mg does not induce an inflammatory phenotype in MEC.

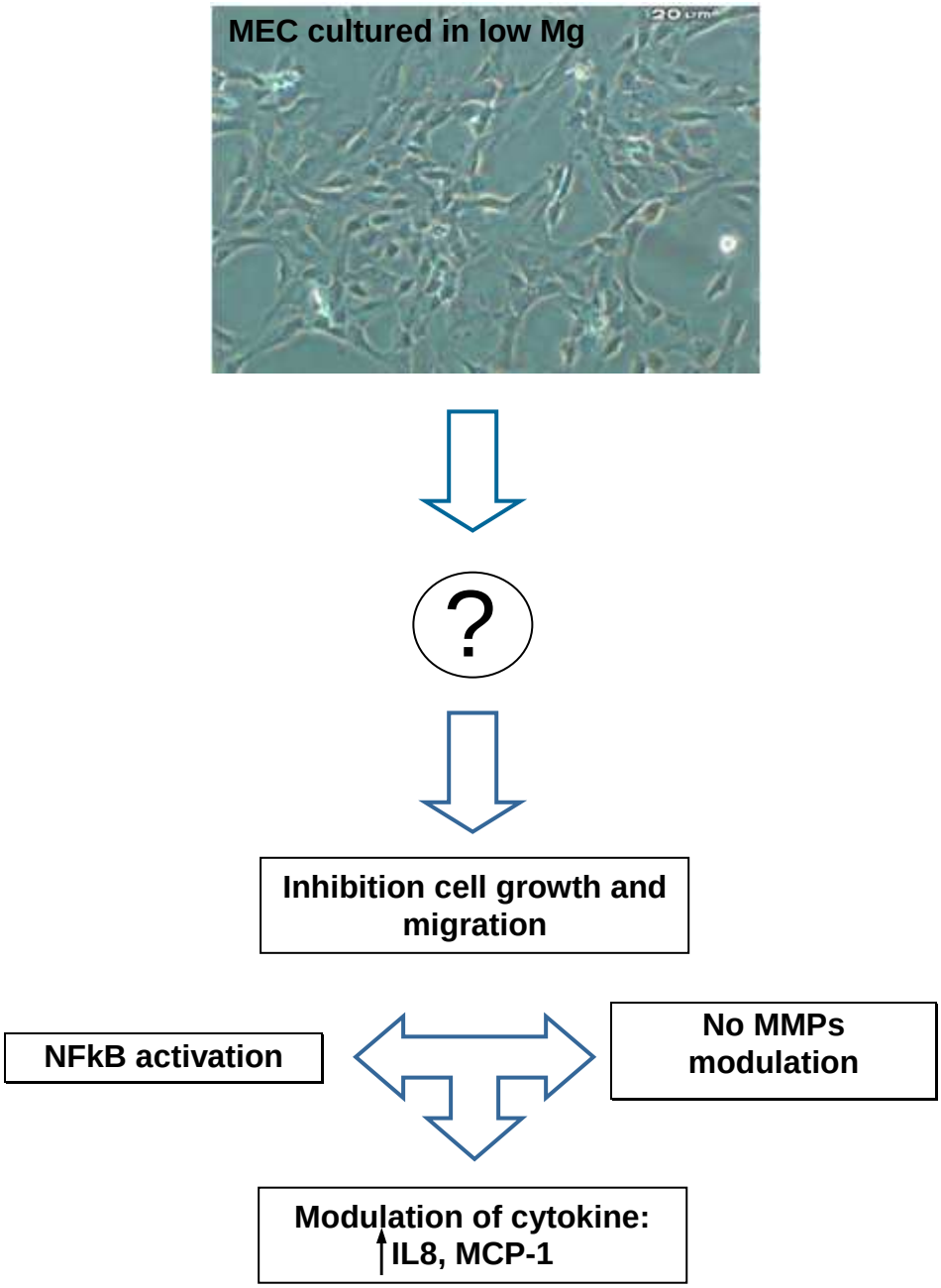

Figure 7.2: Summary of the main results obtained in MEC cultured in low Mg.

7.3 TRPM7 in micro- and macrovascular endothelial cells.

Despite the fact that Mg is an abundant cytosolic cation and that it is so important in various biological processes, there is little information about the transport mechanisms that regulate its homeostasis, especially in vascular cells. It is well known that Mg absorption in the intestine and kidney occurs via paracellular and transcellular pathways. Transporter and exchanger that have been implicated include Na/Mg exchanger, Mg/Ca exchanger and more recently two novel ion channels of the transient receptor potential cation channel superfamily (TRP). These ion channels, TRPM6 and TRPM7, are the principal factors responsabile for the transepithelia transport of Mg and are critical for human health and cell function (Nadler et.al., 2001; Voets et al., 2004). RT-PCR and *in situ* hybridation analyses have demonstrated that TRPM6 is expressed along the entire gastrointestinal tract, in the kidney, intestine and lung (Rondòn LJ. et al., 2008), whereas TRPM7 is ubiquitously expressed and its targeted disruption in different cell lines is lethal, highlighting the critical role of this protein in cell physiology (Touyz RM 2008). *In vitro* studies have shown that TRPM6 and TRPM7 are modulated by intracellular free Mg, Mg-nucleotides and pH. Both channels have an α-kinase domain, with no clearly identified function.
In vascular cells, Mg influx is driven mainly through TRPM7. Some authores showed that an altered cellular Mg homeostasis and abnormal vascular smooth muscle cell function in hypertension may be in part related to defective TRPM7 expression/activity (Touyz RM, 2008). In these cells, ANGII and aldosterone regulate vascular TRPM7 acutely by inducing phosphorylation and chronically by increasing expression at the mRNA and proteins level. Downregulation of vascular TRPM7 by small interference siRNA reduced basal intracellular Mg, ANG II-stimulated [Mg]i transients and attenuated ANG II-mediated VSMC growth. These findings confirmed that TRPM7 is a key regulator of Mg homeostasis and it plays a major role in smooth muscle cell growth (He Y et al., 2005; Touyz RM et al., 2006). Two recent studies in cancer cells and osteoblasts further support the importance of TRPM7 in ion homeostasis and cell proliferation (Abed E. and Moreau R., 2007; Jang J et al, 2007). Very recently, TRPM7 deficient ES cells were shown to be growth inhibited (Ryazanova 2010).

First I evaluated whether MEC and HUVEc expressed TRPMs. I found no expression f TRPM6 by RT-PCR (nor shown). I therefore focused my attention on TRPM7 both in MEC and HUVEC. I found no modulation of TRPM7 amounts in HUVEC rendered quiescent by starvation and then stimulated to re-entry the cell cycle by adding complete growth medium. I also examined TRPM7 levels in sparse, confluent and post-confluent HUVEC and MEC and

observed no modulation. Interestingly, the inflammatory cytokines IL-1α and TNF-α induce an increase in the total TRPM7 amount both HUVEC and MEC cells. I also evaluated the total amounts of TRPM7 in MEC and HUVEc grown in different Mg concentrations (0.1, 1.0 and 5.0 mM Mg). I found that TRPM7 is upregulated in HUVEC cultured in low Mg, probably as an attempt to optimize Mg transport under reduced Mg availavility. Since low Mg induces cytokines in HUVEC and cytokines induce TRPM7, I hypothesize that cytokines mediate low Mg upregulation of TRPM7. Since HUVEC in high Mg downregulate TRPM7, I speculate that this downmodulation protects the cells against an accumulation of the cation. Because of the multiple actions of Mg, an overload of intracellular Mg might impair the coordinated and finely tuned regulation of several intracellular reactions.

TRPM7 regulation by Mg is strikingly different in MEC. Indeed, low Mg reduces the TRPM7 expression in MEC, suggesting the existence of alternative transporters involved in maintaining adequate concentrations of intracellular Mg. It is also possible that in these cells Mg efflux is differently tuned.

TRPM7 supports multiple cellular functions, including cell viability and growth, cell adhesion and migration (Abed E. and Moreau R., 2007; Jang J et al, 2007, Ryazanova 2010). The role of TRPM7 in cell proliferation stems from the evidence that TRPM7 ensures a rapid Mg influx during the G1 phase of the cell cycle (Ryazanova 2010).

I therefore silenced TRPM7 in MEC and HUVEC. As expected MEC silencing TRPM7 were growth retarded and migrated slower than controls. It is possible to conclude that in MEC silencing TRPM7 mimics the effect of low extracellular Mg.

Surprisingly, HUVEC silencing TRPM7 grew faster and migrated more than controls. Because this result was unexpected, I repeated the experiments on primary HUVEC derived from different cords as well as on commercial HUVEC, and consistently obtained the same result. Until now, HUVEC are the only cell type that is stimulated to grow by the downregulation of TRPM7. It is possible to speculate that TRPM7 is not crucial for Mg homeostasis in these cells which might possess other transporters able to maintain adequate concentrations of intracellular Mg.

Conclusions

In this thesis I show that macro- and microvascular endothelial cells are sensitive to low Mg. While the modulation of HUVEC phenotype by low Mg is mediated, at least in part by ROS, in MEC ROS seem not to be involved and the mechanisms contributing to altering MEC behaviour *in vitro* have not been clarified yet. In general, my studies suggest that low Mg promotes a proatherogenic phenotype in HUVEC, and it affects MEC impairing some pivotal events ofangiogenesis. Interestingly, the regulation and the role of the Mg transporter TRPM7 is markedly different in the two cell types.

My results suggest a more cautious interpretation of *in vitro* data, which should result in a stronger consideration of endothelial cell type-specific effects. Results of studies based on a given endothelial cell type should not be transferred uncritically to other types of endothelial cells.

8. REFERENCES

A

- Abed E and Moreau R. Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation. Cell Prolif 40: 849-865, 2007.
- Abed E and Moreau R. Importance of melastatin-like transient receptor potential 7 and magnesium in the stimulation of osteoblast proliferation and migration by platelet-derived growth factor. Am J Physiol Cell Physiol 297: C360-C368, 2009.
- Adachi M, Nara Y, Mano M, Ikeda K, Horie R, Yamori Y. Intralymphocytic free calcium and magnesium in stroke-prone spontaneously hypertensive rats and effects of blood pressure and various antihypertensive agents. Clin Exp Pharmacol physiol 20: 587-593, 1993.
- Agus Z.S. Hypomagnesemia. J Am Soc Nephrol. 10:1616-22,1999.
- Alp NJ and Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler Thromb Vasc Biol 24(3): 413-20, 2004.
- Arnaud M.J. Update on the assessment of magnesium status. Br J Nutr 99 Suppl 3, S24-36, 2008.
- Altura BT, Brust M, Bloom S, Barbour RL, Stempak JG, Altura BM. Magnesium dietary intake modulates blood lipid levels and atherogenesis. Proc natl Acad Sci USA 87(5) : 1840-4,1990.
- Altura Burton M., Gebrewold A., Zhang A. and Altura B. Low extracellular ions induce lipid peroxidation and activation of nuclear factor-kappa B in canine cerebral vascular smooth muscle: possible relation to traumatic brain injury and strokes. Neuroscience letters 341: 189-192, 2003.

B

- Babior BM. NADPH oxidase. Curr Opin Immunol 16(1):42-7, 2004.
- Baldoli E, Leidi M and Maier JAM. Downregulation of HD-PTP by high magnesium concetntration : novel insights into magnesium-induced endothelial migration. Magnes Res 23(3): 119-25, 2010.
- Bara, M., Guiet-Bara, A., and Durlach J. A qualitative theory of the screening-binding effects of magnesium salts on epithelial cell membranes: a new hypothesis. Magnes Res2, 243-248, 1989.
- Barnes PJ, Karin M. Nuclear factor-kappa B: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 336(15):1066-71,1997.
- Begon S, Alloui A, Eschalier A, Mazur A, Rayssiguier Y, Dubray C. Assessment of the relationship between hyperalgesia and peripheral inflammation in magnesium-deficient rats. Life Sci 70(9): 1053-63, 2002.
- Belin RJ, He K. Magnesium physiology and pathogenic mechanisms that contribute to the development of the metabolic syndrome. Magnes Res 20(2) 107-29, 2007.
- Bernardini D, Nasulewich A., Mazur A., Maier JAM. Magnesium and microvascular endothelial cells: a role in inflammation and angiogenesis. Front Biosci 10, 1177-1182, 2005.

- Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. J Physiol 16;555: 589-606, 2004.
- Berthelot A, Arnaud M, Reba A and Henrotte J (2004). “Le Magnesium”. United Kingdom.
- Berkelhammer C. and Bear R.A. A clinical approach to common electrolyte problems: Hypomagnesemia. Can Med Assoc J 132, 360-8, 1985.
- Bolognese A, Correale G, Manfra M, Lavecchia A, Novellino E, Pepe S. Antitumor agents. 5 synthesis, structure-activity relationships and biological evaluation of dimethyl-5H-pyridophenoxazin-5-ones, and 5h-benzopyridophenoxazin-5-ones with potent antiproliferative activity. J Med Chem 49(17): 5110-8, 2006.
- Brannan PG, Vergne-marini P, Pak CY, Hull AR, Fordtran JS. Magnesium absorption in the human small intestine. Results in normal subjects, patients with chronic renal disease, and patients with absorptive hypercalciuria. J Clin Invest. 57: 1412-8, 1976.
- Bukoski RD. Reactive oxygen species: the missing link between magnesium deficiency and hypertension? J Hypertens 20(11): 2141-3, 2002.
- Bui DM, Gregan J, Jarosch E, Ragnini A, Schweyen RJ. The bacterial magnesium transporter CorA can functionally sybstituite fornitsb putative homologue Mrs2p in the yeast inner mitochondrial membrane. J Biol Chem 274: 20438-20443, 1999.
- Bussière FI, Gueux E, Rock E, Mazur A, Rayssiguier Y. Protective effect of calcium deficiency on the inflammatory response in magnesium-deficient rats. Eur J Nutr 41: 197-202, 2002.

C

- Cai H., Harrison DG. Endothelial dysfunction in cardiovascular diseases the role of oxidant stress. Circ Res 87:840-844, 2000.
- Cai Hua. Hydrogen peroxide regulation of endothelial function: Origins, mechanisms, and consequences. Cardiovascular Research 68: 26-36, 2005.
- Callera GE, He Y, Yogi A, Montezano AC, Paravicini T, Yao G, Touyz RM. Regulation of the novel Mg2+ transporter transient receptor potential melastatin 7 (TRPM7) cation channel by bradykinin in vascular smooth muscle cells. J Hypertens 27(1):155-66, 2009.
- Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. Arterioscler Thromb Vasc Biol 20(7):1716-23, 2000.
- Chabrashvill T, Tojo A, Onozato ML, Kitiyakara C, Quinn MT, Fujita T, Welch WJ, Wilcox CS. Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. Hypertension 39(2):269-74, 2002.
- Chetan P, Dharam P, Chaudhary, Devi D, and Bansal M. Effect of magnesium supplemetation on oxidative stress in alloxanic diabetic rats. Magnesium Res 16(1), 13-19, 2003.
- Chollet D, Franken p, raffin Y, henrotte JG, Widmer J, Malafosse A, Tafti M. Magnesium involvement in slee: genetic and nutritional models. Behav Genet 31: 413-25, 2001.
- Chi, J. T., Chang, H. Y., Haraldsen, G., Jahnsen, F. L., Troyanskaya, O. G., Chang, D. S., Wang, Z., Rockson, S. G., van de Rijn, M., Botstein, D., Brown, P. O. Endothelial cell

diversity revealed by global expression profiling. Proc. Natl. Acad. Sci. USA 100**,** 10623 – 10628, 2003.

- Chiueh CC. Neuroprotective properties of nitric oxide. Ann N Y Acad Sci 890: 301-11, 1999.
- Cines DB, Pollak ES, Buck CA, Lo Scalzo J, *et al*. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood; 91:3527-3561, 1998.
- Clapham DE. Sorting out MIC, TRP, and CRAC ion channels. J Gen Physiol 120: 217-220, 2002.
- Clark K, Langeslag M, Van Leeuwen B, Ran L, Ryazanov AG et al. TRPM7, a novel regulator of actinomyosin contractility and cell adhesion. EMBO J 25: 290-301, 2006.
- Classen CU, Abele C, Schimatschek HF, Friedberg KD, Classen HG, Haubold W. Erythema formation in magnesium-deficient albino rats. A non invasive model for the screening of anti-inflammatory agents and oral mineral supplements. Arzneimittel 43: 672-5.
- Cole D.E., and Quamme G.A. Inherited disordes of renal magnesium handling.J Am Soc Nephro 11, 1937-47, 2000.
- Crosby V, Wilcock A, lawson N, Cordoran R. The importance of low magnesium in palliative care. Palliat Med 14: 544, 2000.

D

- Dai L.J., Ritchie G., Kerstan D., Kang H.S., Cole D.E. and Quamme G.A. Magnesium transport in the renal distal convoluted tubule. Physiol Rev 81,51-84, 2001.
- Davenport G.M., Boling J.A. and Gay N.Bioavailability of magnesium in beef cattle fed magnesium oxide or magnesium hydroxide. J Anim Sci 68, 3765-72, 1990.
- Deheinzelin D, Negri EM, Tucci MR, salem MZ, da Cruz VM, Oliveira RM, Nishimoto IN, Hoelz C. Hypomagnesemia in critically ill cancer patients: a prospective study of predictive factors. Braz J Med Biol Res 33: 1443-8, 2000.
- Demeuse P, Penner R and Fleig A. TRPM7 channel is regulated by magnesium nucleotides via its kinase domain. J Gen Physiol 127: 421-434, 2006.
- Dorup I and Clausen T. Correlation between magnesium and potassium contents in muscle: role of Na(+)-K+ pump. Am J Physiol 264: C457-63, 1993.
- Dorovkov MV, Ryazanov AG. Phosphorylation of annexin 1 by TRPM7 channel-kinase. J Biol Chem 279: 50643-50646, 2004.
- de Rouffignac C. and Quamme G. Renal magnesium handling and its hormonal control. Physiol Rev 74, 305-22, 1994.
- Viemann D, Goebeler M, Schmid S, Nordhues U, Klimmek K, Sorg C, Roth J. TNF induces distinct gene expression programs in microvascular and macrovascular endothelial cells. J. Leukoc. Biol. 80: 174–185, 2006.
- Dröge W. Free radicals in the physiological control of cell function. Physiol rev 82(1): 47-95, 2002.
- Duncan LM, Deeds J, Hunter J, Shao J, Holmgren LM, Woolf EA, Tepper RI, Shyjan AW. Down-regulation of the novel gene melastatin correlates with potential for melanoma matastasis. Cancer Res 58: 1515-1520, 1998.

- Dunn M.J. and Walser M. Magnesium depletion in normal man. Metabolism 15, 884-95, 1966.

F

- Fawcett W.J, Haxby EJ and Male DA. Magnesium: physiology and pharmacology. Br J Anaesth 83, 302-20, 1999.
- Ferré S, Mazur A, Maier JAM. Low-magnesium induces senescent features in cultured human endothelial cells. Magnes Res 20:66-71, 2007.
- Flatman PW. Magnesium transport across cell membranes. J Membr Biol 80, 1-14, 1984.
- Fleig A and Penner R. The TRPM ion channel subfamily: molecular, biophysical and functional features. Trends Pharmacol Sci 25: 633-639, 2004.
- Fine K.D, Santa Ana, Porter J.L, and Fortran. Intestinal absorption of magnesium from food and supplements.J Clin Invest 88, 396-402, 1991.

G

- Gabbe SG. A preliminary report on the intravenous use of magnesium sulphate in puerperal eclampsia. Am J Obstet Gynecol 174:1390-1391, 1996.
- Galis ZS and Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 90: 251-262, 2002.
- Garfinkel L., and Garfinkel D. Magnesium regulation of the glycolytic pathway and the enzymes involved. Magnesium 4, 60-72, 1985.
- Gazmuri RJ et al., 2001
- Geiszt M. NADPH oxidases: new kids on the block. Cardiovasc Res 71(2):289-99, 2006.
- Ghafourifar P and Cadenas E. Mitochondrial nitric oxide synthase. Trends Pharmacol 26(4): 190-195, 2005.
- Gold ME, Buga GM, Wood KS, Byrns RE, Chadhuri G and Ignarro LJ. Antagonistic modulatory roles of magnnesium and calcium on release of endothelium-derived relaxing factor and smooth muscle tone. Circ Res 66: 355-366,1990.
- Griendling KK, Sorescu D, Lassegue B, Uschio-Fukai M. ,odulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb vasc Biol 20(10):2175-83, 2000.
- Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ res 86(5):494-501, 2000.
- Griendling KK. NADPH oxidases: new regulators of old functions. Antioxid Redox Signal 8(9-10):1443-5, 2006.
- Grimm C, Kraft R, Sauerbruch S, Schultz G, Harteneck C. Molecular and functional characterization of the melastatin-related cation channel TRPM3. J Biol Chem 278(24):21493-501, 2003.
- Grote K, Flach I, Luchtefeld M, Akin E, Holland SM, Drexler H and Schieffer B. Mechanical stretch enhances mRNA expression and proenzyme release of matrix

metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxigen species. Circ res 92: e80-e86, 2003.

- Guerrero-Romero Fernando, Martha Rodriguez-Moràn. Hypomagnesemia, oxidative stress, inflammation, and metabolic syndrome. Diabets/metabolism Res Rev 22: 471-476, 2006.
- Gurjar MV, Deleon J, Sharma RV, and Bhalla RC. Role of reactive oxygen species in IL-1 beta-stimulated sustained ERK activation and MMP-9 induction. Am J Physiol Heart Circ Physiol 281: H2568- H2574, 2001.
- Gunther T, Vormann J, Hollriegl V. Isoproterenol-induced Mg uptake in liver. FEBS Lett 307: 333-336, 1992.
- Gunther T. Na^+/Mg^+ antiport in non-erythrocyte vertebrate cells. Magnes Res 20: 89-99, 2007.
- Gunther T. Comment on the number of Mg2+ -activated enzymes. Mag Res 21: 185-7, 2008.
- Guzik TJ, korbut R, Adamek-Guzik t. Nitric oxide and superoxide in inflammation and immune regulation. J Physiol Pharmacol 54: 469-87, 2003.
- Guzik TJ, Sadowski J, Kapelak B, Jopek A, Rudzinski P, Pillai R, Korbut R, Channon KM. Systemic regulation of vacsular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. Arterioscler Thromb Vasc Biol 24(9):1614-20, 2004.

H

- Hackett SF, Friedman Z, Campochiaro PA. Cyclic 3',5'-adenosine monophosphate modulated vascular endothelial cell migration in vitro. Cell Biol Int Rep 11: 279-287, 1987.
- Halliwell B, Zhao K, Whiteman M. Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. Free Radic Res 31(6): 651-69, 1999.
- Hancock JT, Salisbury V, Ovejero-Boglione MC, Cherry R, Hoare C et al. Antimicrobial properties of milk: dependence on presence of xanthine oxidase and nitrite. Antimicrob Agents Chemother 46(10): 3308-3310, 2002.
- Hans C.P., Chaudhary D.P., and Bansal D.D. Effects of magnesium supplementation on oxidative stress in alloxanic diabetic rats. Magnesium Research 16, 13-19, 2003.
- Hardwick L.L, Jones M., Brautbar N. and Lee D.B. Magnesium absorption: machanisms and the influence of vitamine D, calcium and phosphate.J Nutr 121, 13-23, 1991.
- Henrotte J.G., Pla M. and Dausset J. HLA and H-2-associated variations of intra and extracellular magnesium content.Pro Natl Acad Science USA 87,1894-8, 1990.
- Huang S. and Kuo E. Mechanism of hypokalemia in magnesium deficiency. J Am Soc Nephrol 18, 2649-52, 2007.

I

- Ignarro L.J. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annual Rev Pharmacol. Toxicol 30, 535-560, 1990.
- Ionue R, Jensen LJ, Shi J, Morita H, Nishida m, Honda A, Ito Y. transient receptor potential channels in cardiovascular function and disease. Circ Res 99: 119-131, 2006.

- Iseri L.T, and French J.H. Magnesium: nature's physiologic calcium blocker. Am Heart J 108, 188-193, 1984.

J

- Jeong SM, Hahn KD, Shin JW, Leem JC, Lee C, and Han SM. Changes in magnesium concentration in the serum and cerebrospinal fluid of neurophatic rats. Acta Anaesthesiol Scand 50: 211-216, 2006.
- Jiang J, Newell, E.W., Schlichter LC. Regulation of a TRPM7 like current in rat brain microglia. J Biol Chem 278: 42867-76, 2003.

K

- Kayne L.H., and Lee D.B. Intestinal magnesium absorption. Miner Electrolyte Metab 19, 210-217, 1993.
- Kanazawa I., Yamamoto M., Yamaguchi T., Yamamuchi M., Yano S. And Sugimoto T. A case di magnesium deficiency associated with insufficient parathyroid hormone action and severe osteoporosis. Endocr J 54, 935-40, 2007.
- Kantorovich V., Adams J.S., Gaines J. E., Guo X.,Pandian M.R., Cohn D.H. and Rude R.K. Genetic heterogeneity in familial renal magnesium wasting. J Clin Endocrinal Metab 87, 612-617, 2002.
- Karbach U. and Feldmeier H. New clinical and experimentals aspects of intestinal magnesium transport. Magnesium Res 4: 9-22, 1991.
- Karin M, Takahashi T, Kapahi P, Delhase M, Chen Y, Makris C, Rothwarf D, Baud V, Natoli G, Guido F, Li N. Oxidative stress and gene expression: the AP-1 and NF-kappa B connections. Biofactors. 15(2-4):87-9, 2001.
- Khan LA, Alam AM, AliL, Goswami A, Hassan Z, Sattar S, Banik NG, and Khan AK. Serum and urinary magnesium in young diabetics subjects in Bangladesh. Am J Clin Nutr 69: 70-73, 1999.
- Killilea DW and Ames BN. Magnesium deficiency accelerates cellular senescence in cultured human fibroblasts. Proc Natl Acad Sci USA 105(15):5768-73, 2008.
- Killilea and Maier JAM. A connection between magnesium deficiency and aging: new insights from cellular studies. Nutrition and metabolism center 21(2):77-82, 2008.
- Klatt P, Lamas S. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Eur J Biochem 267(16): 4928-44, 2000.
- Ko EA, Park WS, Earm YE. Extracellular Mg blocks endothelin-1 induced contraction through the inhibition of non-selective cation channels in coronary smooth muscle. Pluger Arch 451: 204-211, 2005.
- Kolisek M, Zsurka G, Samaj J, Weghuber J, Schweyen RJ, Schweigel M. Mrs2 is an essential component of the major electrophoretic Mg influx system in mitochondria. EMBO J 22: 1235-1244, 2004.
- Konrad M. and Weber S. Recent advances in molecular genetics of hereditary magnesium-losing disorders. J Am Soc Nephrol 14: 249-60, 2003.

- Konrad M, Schaller A, Seelow D et al. Mutations in the tight-junction gene claudin 19 (CLDN19) are associated with renal magnesium wasting, renal failure, and severe ocular involvement. Am J Hum Genet 79: 949-957, 2006.
- Kosaka C, Sasaguri T, Ishida A, Ogata J. Cell cycle arrest in the G2 phase induced by phorbol ester and diacylglycerol in vascular endothelial cells. Am. J. Physiol. 270 C170-178, 1996.
- Kramer JH, Misik V, Weglicki WB. Magnesium-deficiency potentiates free radical production associated with post-ischemic injury to hearts: vitamin E affords protection. Free Rad Biol Med 16: 713-723, 1994.
- Kramer JH, Phillips TM, weglicki WB.magnesium-deficiency enhanced post ischemic myocardial injury is reduced by substance P receptor blockade. J Mol Cell Cardiol 29: 97-110, 1997.
- Kramer JH, Spurney C, Mak I-T et al. Echocardiography reveals progressive cardiac dysfunction in Mg-deficient rats. J Investigative Med 57:318, 2009.
- Kramer JH, Spurney C, Micaela Iantorno, Tziros C, Mak I-T et al. Neurogenic inflammation and cardiac dysfunction due to hypomagnesemia.The American journal of the Medical Sciences 338: 22-27, 2009.

L

- Lamalice L, Le Boeuf F, Huot J. Endothelial migration during angiogenesis. Circ Res 100: 782-794, 2007.
- Langeslag M, Clark K, Moolenaar WH, van Leeuwen, F.N., and Jalink K. Activation of TRPM7 channels by phospholipase C-coupled receptor agonists. J Biol Chem 282: 232-9, 2007.
- Lang I, Pabst MA, Hiden U, Blaschitz A, Dohr G, Hahn T, et al. Heterogeneity of microvascular endothelial cells isolated from human term placenta and macrovascular umbilical vein endothelial cells. Eur J Cell Biol 82: 163-173, 2003.
- Lapidos KA, Woodhouse EC, Kohn EC, Masiero L. $Mg^{(++)}$-induced endothelial cell migration: substratum selectivity and receptor-involvement. Angiogenesis 4: 21-28, 2001.
- Laurant P. and Berthelot A. Endothelin-1 induced contraction in isolated aortae from normotensive and DOCA-salt hypertensive rats: effect of magnesium.Br J Pharmacol 119: 1367-1374, 1996.
- Laurant P, and Touyz, R.M. Physiological and pathophysiological role of magnesium in the cardiovascular system:implications in hypertension. J Hypertens 18:1177-91, 2000.
- Li M, Du J, Jiang J, Ratzan, W.J., Su, L.T., Runnels, L.W. and Yue L. Molecular determinants of Mg^{2+} and Ca^{2+} permeability and Ph sensitivity in TRPM6 and TRPM7. J Biol Chem, 2007.
- Liao F, Folsom AR, Brancati FL. Is low magnesium concentration a risk factor for coronary heart disease? The Atherosclerosis Risk in Communities (ARIC) study. Am heart J 136: 480-90, 1998.
- Littlefield NA, Hass BS, L.J. McGarry, S.M. Morris. Effect of magnesium on the growth and cell cycle of transformed and non-transformed epithelial rat liver cells in vitro. Cell Biol. Toxicol. 7: 203-214, 1991.

M

- Maier JAM. Low magnesium and atherosclerosis: an evidence-based link. Mol Aspects Med 24: 137-46, 2003.
- Maier JAM, Malpuech-Brugère C., Zimowska W., Rassiguier Y and Mazur A. (2004). Low magnesium promotes endothelial cell dysfunction: implications for atherosclerosis, inflammation and thrombosis. Biochim Biophys Acta 24;1689(1): 13-21, 2004.
- Maier JAM, Nasulewicz-Goldeman A, Simonacci N, Boninsegna A, Mazur A, Wolf FI. Insights into the mechanisms involved in magnesium-dependent inhibition of primary tumor growth. Nutr. Cancer 59(2):192-198, 2007.
- Mayer ML, Westbrook GL, and Guthrie PB. Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurones. Nature 309: 261-263, 1984.
- Mandon B, Siga E, Roinel N and deRouffignac C. Ca, Mg and K transport in the cortical and medullary thick ascending limb of the rat nephron: influence of transepithelial voltage. Pflugers Arch 424,558-60, 1993.
- Manju L, Nair RR. Magnesium deficiency augments myocardial response to reactive oxygen species. Can J Physiol Pharmacol. 84: 617-24, 2006.
- Mak IT, Dickens BF, Komarov AM, Wagner TL, Phillips TM, Weglicki WB. Activation of the neutrophil and loss of plasma glutathione during Mg-deficiency-modulation by nitric oxide synthase inhibition. Mol Cell Biochem 176(1-2): 35-9, 1997.
- Mak IT, Kramer JH, Weglicki WB. Suppression of neutrophil and endothelial activation by substance P receptor blockade in the Mg-deficient rat. Magnes Res 16(2): 91-97, 2003.
- Matlung HL, Bakker EN, VanBavel E. Shear stress, reactive oxygen species, and arterial structure function. Antioxid Redox Signal 11(7): 1699-709, 2009.
- Matsuda H. Magnesium gating of the inwardly rectifying K channel. Annu Rev Physiol 53: 289-298, 1991.
- Mazur A., Maier JA, Rock E., Gueux E., Nowacki W. and Rayssiguier Y. Magnesium and the inflammatory response: potential physiopathological implications. Archives Biochem Biophys 458:48-56, 2007.
- McCance, K. L., & Huether, S. E. (2006). Pathophysiology: The biologic basis for disease in adults and children (5th ed.). Chicago.
- McKemy DD, Neuhausser WM, Julius D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416: 52-58, 2002.
- McNeill MS, Paulsen J, Bonde E, burnight MY Hsu RA Cornell. Cell death of melanophores in zebrafish TRPM7 mutant embryos depends on melanina synthesis. J Invest Dermatol 127: 2020-2030, 2007.
- Michailova A and McCulloch AD. Effects of Mg^{2+}, pH and PCr on cardiac excitation metabolic coupling. Magnesium Research 21,16-28, 2008.
- Michaelsson K, Holmberg L., Mallmin H., Wolk A., Bergstrom R. and Ljunghall S. Diet, bone, mass and osteocalcin a cross-sectional study. Calcif Tissue Int 57: 86-93,1995.
- Miller DM., Buettner GR, and Aust SD. Transition metals as catalysts of ″autoxidation″ reactions. Free Radic Biol Med 8 : 95-108, 1990.
- Montell C. The TRP superfamily of cation channels. Sci STKE, 2005.

- Monteilh-Zoller MK, Hermosura MC, Nadler MJ, scharenberg AM, Penner R, Fleig A. TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions.J Gen Physiol 121: 49-60, 2003.

N

- Naderi AS. and Reilly RF. Hereditary etiologies of hypomagnesemia. Nat Clin Pract Nephrol 4, 80-9, 2008.
- Nadler J.L., Buchanan T., Natarajan R., Antonipillai I., Bergman R. And Rude R. magnesium deficiency produces insulin resistance and increased thromboxane synthesis. Hypertension 21, 1024-9, 1993.
- Nadler J. and Rude R. Disorders of magnesium metabolism. Endocrinol Metab Clin North Am 24(3), 623-41, 1995.
- Navarro-Gonzàlez JF, Mora-Fernàndez C, Garcìa-Pérez J. Clinical implications of disordered magnesium homeostasis in chronic renal failure and dialysis. Semin Dial 22(1): 37-44, 2009.
- Nielsen FH. Magnesium, inflammation and obesity in chronic disease. Nutr Rev 68(6): 333-340, 2010.
- Nilsson, Hoffmann I. Cell cycle regulation by the Cdc25 phosphatase family, Prog. Cell Cycle Res. 4:107-114, 2000.

O

- Ohara Y, Peterson TE, Harrison DG.Dietary correction of hypercholesterolemia in the rabbit normalizes endothelial superoxide anion production. Circulation 92: 898-903, 1995.

P

- Pahl HL. Activators and target genes of Rel/ NF-kappaB transcription factors. Oncogene 18: 6853-66, 1999.
- Panus PC, Wright SA, Chumley PH, Radi R, Freeman BA. The contribution of vascular endothelial xanthine dehydrogenase/oxidase oxigen mediated cell injury. Arch Biochem Biophys, 294(2): 695-702, 1992.
- Paolisso G, Sgambato S., Giuliano D., Torella R., Varricchio M. Scheen A.J., D'onofrio F. and Lefebvre P.J. Impaired insulin-induced erythrocyte magnesium accumulation is correlated to impaired insulin-mediated glucose disposal in type-2 (non-insulin-dependent) diabetic patients. Diabetologia 31: 910-915, 1988.
- Pedersen SF, Owsianik G, Nilius B. TRP channels: an overview. Cell Calcium 38: 233-252, 2005.
- Peter Libby, Paul M Ridker, Göran K. Hansson. Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol 2009; 54:2129–38.

Q

- Quamme G.A. Magnesium homeostasis and renal magnesium handling. Miner Electrolyte Metab 19,218-25, 1993.

R

- Rajagopalan S, Ping Meng X, Ramasamy S, Harrison DG and galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix matalloproteinases in vitro. J Clin Invest 98 (11):2572-2579, 1996.
- Rayssiguier Y., Bussiere F, Gueux E, Rock E, Mazur A. Advances in magnesium research: Nutrition and health, John libbey, London, 2001, pp.277-283.
- Ray R, Shah AM. NADPH oxidase and endothelial cell function. Clin Sci 109: 217-226, 2005.
- Rasmussen H.Sandvad, Aurup P, Goldstein K, McNair P, Mortensen B, Larsen O.G., Lawaetz H. Infleuence of magnesium substitution therapy on blood lipid composition in patients with ischemic heart disease. 149(5):1050-1053, 1989.
- Ravn HB, Korsholm TL, Falk E. Oral magnesium supplementation induces favorable antiatherogenic changes in ApoE- deficient mice. Atheroscler. thromb. Vasc Biol 21: 858-862, 2001.
- Rescher U, Ruthe D, Ludwing C, Zobiack N, Gerke V. Annexin 2 is a phosphatidylinositol (4,5)-bisphosphate binding protein recruited to actin assembly sites at cellular membranes. J Cell Sci 117 : 3473-80, 2004.
- Ridnour LA, Thomas DD, Mancardi D, Espey MG, Miranda KM, Paolocci N, feelish M, Fukuto J, Wink DA. The chemistry of nitrosative stress induced by nitric oxide and reactive nitrigen oxide species. Putting perspective on stressful biological situations. Biol Chem 385(1): 1-10, 2004.
- Risco F, Traba ML. Influence of magnesium on the in vitro synthesis of 24,25-dihydroxyvitamin D3 and 1 alpha, 25-dihydroxyvitamin D3. Magnes Res 5: 5-14, 1992.
- Robb EL, Page MM, Wiens BE, Stuart JA. Molecular mechanisms of oxidative stress resistance induced by resveratrol: specific and progressive induction of MnSOD. Biochemical and Biophysical Research Communications 367 (2): 406–12, 2008.
- Rodrìguez-Moran M. and Guerrero-Romero F. Oral magnesium supplementation improbe insulin sensitivity and metabolic control in type 2 diabetic subjects: a randomized double-blind controlled trial. Diabetes Care 26, 1147-52, 2003.
- Romani and Scarpa. Regulation of cellular magnesium. Front biosci 1, D720-34, 2000.
- Romani AM. Magnasium homeostasis in mammalian cells. Front Biosci 12, 308-31, 2007.
- Rondòn LJ., Tiel Groenestege WM., Rayssiguier Y and Mazur A. relationship between low magnesium status and TRPM6 expression in the kidney and large intestine. Am J Physiol Regal Integr Comp Physiol 294: 2001-2007, 2008.
- Ross R. Atherosclerosis is an inflammatory disease. Am Heart J 138: S419-20, 1999.
- Rubin H, Magnesium: The missing element in molecular views of cell proliferation control, Bioessays 27 (2005) 311-320.

- Rude RK. and Ryzen. TmMg and renal Mg threshold in normal man and in certain pathophysiologic conditions. Magnasium, 5:273-281, 1986.
- Rude RK and Ryzen E. Ventricular fibrillation during antiarrhythmic drug therapy. N Engl J Med 320 : 537-8, 1989.
- Rude RK, Stephen A, Nadler J. Determination of red blood cell intracellular free magnesium by nuclear magnetic resonance as an assessment of magnesium depletion. Magnes Trace Elem 10 : 117-21, 1991.
- Rude RK. Magnesium Deficiency :a cause of heteogenous disease in humans. J Bone and Mineral Research, 13:749-58, 1998.
- Rude RK, Stephen A, Nadler J. Determination of red blood cell intracellular free magnesium by nuclear megnetic resonance as an assessment of magnesium depletion. Magnes trace Elem, 10: 117-121, 1991.
- Rude RK, Kirchen M.E., Gruber H.E., Meyer M.H., Luck J.S. and Crawford D.L. Magnesium deficiency-induced osteoporosis in the rat: uncoupling of bone formation and bone resorption. Magnes Res;12, 257-67, 1999.
- Rude RK and Gruber H.E. Magnesium deficiency and osteoporosis: anima and human observations.J Nutr Biochem 15, 710-716, 2004.
- Rude R.K, Gruber H.E., Wei L.Y., Frausto A. and Mills B.G. Magnesium deficiency: effect on bone and mineral matabolism in the mouse. Calcif Tissue Int 72, 32-41, 2003.
- Runnels LW, Yue L, Clapham DE. The TRPM7 channel is inactivated by PIP_2 hydrolysis. Nat Cell Biol 4: 329-336, 2002.
- Ryazanova LV, Rondon LJ, Zierler S, Hu Z, Galli J, Yamaguchi TP, Mazur A, Fleig A, Ryazanov AG. TRPM7 is essential for Mg(2+) homeostasis in mammals. Nat Commun 1(8):109, 2010.

S

- Sapna S., Ranijth S.K and Shivakumar K. Cardiac fibrogenesis in magnesium deficiency: a role for circulating angiotensin II and aldosterone. Am J Physiol Heart Circ Physiol 291, H436-440, 2006.
- Saris N.E., Mervala E., Karppanen H., Khawaja J.A. and Lewenstam A. Magnesium. An update on physiological, clinical and analytical aspects. Clin Chim Acta 294, 1-26, 2000.
- Satake K, Lee JD, Shimizu H, Uzui H, Mitsuke Y, Yue H, Ueda T. Effect of magnesium on prostacyclin synthesis and intracellular free calcium concentration in vascular cells. Magnes Res 17: 20-27, 2004.
- Schachinger V, Zeiher AM. Atherosclerosis associated endothelial dysfunction. 89 Suppl 9:IX 770-4, 2000.
- Schlingmann KP, Weber S, Peters M, Nejsum LN, VitzthumH et al.hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. Nat Genet 31: 166-170, 2002.
- Schlingmann KP, Konrad M., and Seyberth HW. Genetics of hereditary disorders of magnesium homeostasis. Pediatr Nephrol 19, 13-25, 2004.

- Schlingmann K.P, Waldegger S., Konrad M., Chubanov V. and Gudermann T. TRPM6 and TRPM7-Gatekeepers of human magnesium metabolism. Biochimica et Biophysica Acta 1772: 813-821, 2007.
- Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, kurosaki T, Fleig A. Regulation of vertebrate cellular Mg homeostasis by TRPM7. Cell 114: 191-200, 2003.
- Schmitz C, Deason F. and Perraud AL. Moleculare components of vertebrate Mg^{2+} homeostasis regulation. Magnesium Research; 20, 6-18, 2007.
- Schwartz R., Spencer H. and Welsh J.J. Magnesium absorption in human subjects from leafly vegetables, intrinsically labeled with stable 26Mg.Am J. Clin Nutr 39, 571-6, 1984.
- Shechter M. The role of magnesium as antithrombotic therapy. Wien Med Wochenschr 150: 343-7, 2000.
- Shechter M. Magnesium and cardiovascular system. Magnes Res 23(2): 60-72, 2010.
- Shils M.E. Experimentals human magnesium depletion. Medicine (Baltimore) 48,61-85 1969.
- Shivakumar K. and Kumar BP. Magnesium deficiency enhances oxidative stress and collagen synthesis in vivo in the aorta of rats. Int J Biochem Cell Biol 29,1273-8, 1997.
- Silverman RA, Obsorn H, Runge J et al. IV magnesium sulfate in the treatment of acute severe asthma: a multicenter randomized controlled trial.Chest 122,489-497, 2002.
- Simon DB, Lu Y, Choate KA, Velazquez H, Al-Sabban E, Praga M, Casarini G, Bettinelli A et al. Paracellin-1, a renal tight junction protein required for paracellular Mg resorption. Science 285: 103-106, 1999.
- Singh J, Hustler BI, waring JJ, Howarth FC. Dietary and physiological studies to investigate the relationship between calcium and magnesium signalling in the mammalin myocardium. Mol Cell Biochem 176(1-2): 127-34, 1997.
- Solomon R. The relationship between disorders of K and Mg homeostasis. Semin Nephrol 7, 253-262, 1987.
- Soltani N, Keshavarz M, Sohanaki H, Zahedi S, Dehpour AR. Relaxatory effect of magnesium on mesenteric vascular beds differs from normal and streptozotocin induced diabetics rats. Eur J Pharmacol 508: 177-181, 2005.
- Sontia B and Touyz RM. Magnesium transport in hypertension. Pathophysiology 14(3-4): 205-11, 2007.
- Sontia B, Touyz RM. Role of magnesium in hypertension. Arch Biochem Biophys 458(1): 33-9, 2007.
- Sorescu D, Griendling KK. Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure. Congest Heart Fail 8(3): 132-40, 2002.
- Speich M., Bousquet B., and Nicolas G. Reference values for ionized, complexed, and protein-bound plasma magnesium in men and women. Clin Chem 27,246-8, 1981.
- Spiekermann S, Landmesser U, Dikalov S, Bredt M, Gamez G, Tatge H, Reepschlager N, Hornig B, Drexler H, Harrison DG. Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 107(10): 1383-9, 2003.
- Stevens CR, Millar TM, Clinch JG, Kanczler JM, Bodamyli T, Blake DR. Antibacterial properties of xanthine oxidase in human milk. Lancet 356:829-830, 2000.

- Su LT, Agrapito MA, Li M, Simonson WT, Huttenlocher A, Habas R, Yue L, Runnels LW. TRPM7 regulates cell adhesion by controlling the calcium-dependent protease calpain. J Biol Chem 281: 11260-11270, 2006.
- Such and Hille. Electrostatic interaction of internal Mg^{2+} with membrane PIP2 seen with KCNQ K^{+} channels.J Biol Chem 199, 301-5, 2007.
- Suh S.M., Csima A. and Fraser D. Pathogenesis of hypocalcemia in magnesium depletion. Normal end-organ responsiveness to parathyroid hormone. J Clin Invest 50, 2668-78, 1971.
- Szabo C. The pathophysiological role of peroxynitrite in shock, inflammation, and in ischemia-reperfusion injury. Shock 6: 79-88, 1996.
- Swaminathan R. Magnesium metabolism and its disorders. Clin Biochem Rev 24(2): 47-66, 2003.

T

- Takaya, J., Higashino, H., and Kobayashi. Can magnesium act as a second messenger? Current data on translocation induced by various biologically active substances. Magnes Res 13,139-46, 2000.
- Takezawa R, Schmitz C, Demeuse P, Scharenberg AM, Penner R, Fleig A. Receptor mediated regulation of the TRPM7 channel through its endogenous protein kinase domain. Proc Natl Acad Sci USA 101: 6009-6014, 2004.
- Taniyama Y and Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. Hypertension 42: 1075-1081, 2005.
- Touyz RM, Pu Q, He G, Chen X, Yao G, Neves MF,Viel E. Effects of low dietary magnesium intake on development of hypertension in stroke-prone spontaneously hypertensive rats: role of reactive oxygen species. J Hypertens 21: 2221-2223, 2002.
- Touyz RM, Tabet F, Schiffrin EL. Redox-dependent signalling by angiotensin II and vascular remodelling in hypertension. Clin Exp Pharmacol Physiol 30(11).860-6, 2003.
- Touyz RM. and Schiffrin EL. Reactive oxygen species in vascular biology : implication in hypertension. Histochem Cell Biol 122: 339-352, 2004.
- Touyz RM . Vascular remodelling, Retinal Arteries, and Hypertension. Hypertension 50:603-604, 2007.
- Touyz RM. Transient receptor potential melastatin 6 and 7 channels, magnesium transport, and vascular biology: implications in hypertension. Am J Physiol Heart Circ Physiol 294, H1103-H1118, 2008.
- Traber; Atkinson J. Vitamin E, antioxidant and nothing more. Free radical biology & medicine 43(1): 4-15, 2007.

U

- Ulugol A, Aslantas A, Ipci Y, Tuncer A, Hakan Karadag C, Dokmeci I. Combined systemic administration of morphine and magnesium sulfate pain-related behavior in mononeurophatic rets. Brain Res 943: 101-4, 2002.

V

- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metal and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160(1): 7-40, 2006.
- Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. Circ J 73:595-601, 2009.
- Vernon W. The role of magnesium in nucleic-acid and protein metabolism. Magnesium 7: 234-248, 1988.
- Vitale JJ, White PL, Nakamura M, Hegsted DM, Zamcheck N, hellerstein EE. Interrelationships between experimental hypercholesteremia, magnesium requirement, and experimental atherosclerosis. J Exp Med 106(5): 757-66, 1957.
- Vitale JJ, nakamura m, Hegsted DM. The effect of magnesium deficiency on oxidative phosphorylation. J Biol Chem 228: 573-6, 1957.
- Venkatachalam K and Montell C. TRP channels. Annu Rev Biochem 76: 387-417, 2007.

Z

- Zhou Q, Olinescu RM, Kummerow FA. Influence of low magnesium concentrations in the medium on the antioxidant system in cultured human arterial endothelial cells. Magnesium Research 12(1): 19-29, 1999.

W

- Weglicki WB, Phillips TM, Freedman AM, Cassidy MM, Dickens BF. Magnesium–deficiency elevates circulating levels of inflammatory cytokines and endothelin. Mol Cell Biochem 110: 169-173, 1992.
- Weglicki WB, Mak IT, Phillips TM. Blockade of cardiac inflammation in Mg deficiency by substance P receptor inhibition. Circ Res 74: 1009-13, 1994.
- Weglicki WB, Mak IT, Kramer JH, Dickens BF, Cassidy MM, Stafford RE, Philips TM. Role of free radicals and substance P in magnesium deficiency. Cardiovascular Res 31: 677-682, 1996.
- Weglicki WB, Quamme G, Tucker K, Haigney M, Resnick L. Potassium, magnesium, and electrolyte imbalance and complications in disease management. Clin Exp Hypertens 27: 95-112, 2005.
- White RE, Hartzell HC (1989). Magnesium ions in cardiac function. Biochem pharma 38: 859-867.
- Wolf F, Trapani V. Cell (patho)physiology of magnesium. Clin Sci 114(1): 27-35, 2008.
- Wolf FI, Cittadini A. Magnesium in cell proliferation and differentiation. Front Biosci 4: D607-17, 1999.
- Wolin MS. Reactive oxygen species and vascular signal transduction mechanisms. Microcirculation 3(1): 1-17, 1996.

Y

- Yang XR, Lin, M.J., McIntosh, L.S., and Sham JS. Functional expression of transient receptor potential melastatin- and vanilloid-related channels in pulmonary arterial and aortic smooth muscle. Am J Physiol Lung Cell Molec Physiol 290, L1267-76, 2006.

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