Endothelial cells and magnesium: implications in atherosclerosis

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ABSTRACT
There is no doubt that the functional and structural integrity of the endothelium is critical in maintaining vascular homoeostasis and in preventing atherosclerosis. In the light of epidemiological and experimental studies, magnesium deficiency is emerging as an inducer of endothelial dysfunction. In particular, data on the effects of low extracellular magnesium on cultured endothelial cells reinforce the idea that correcting magnesium homoeostasis might be a helpful and inexpensive intervention to prevent and treat endothelial dysfunction and, consequently, atherosclerosis.

INTRODUCTION
Despite considerable advances over the last decades, cardiovascular diseases exact a very high toll as the leading cause of death in Western societies. This is mainly the result of the increasing prevalence of atherosclerosis, related to the aging of the population, the increase in diabetes and obesity, unhealthy diets, and the under-recognition and under-control of different risk factors for atherosclerosis, in particular hyperlipaemia and hypertension [1].

Atherosclerosis is considered to be a chronic inflammatory disease resulting from the interaction between modified lipoproteins, monocyte-derived macrophages, and endothelial and smooth muscle cells [2]. Initially, this inflammatory process results in the thickening of the arterial wall and then in the formation of complex lesions, the atherosclerotic plaques, through a process that occurs slowly and silently over decades. The plaques protrude in the arterial lumen and, when complicated, cause myocardial infarction, stroke and gangrene [1,2]. Atherosclerosis is initiated upon the modulation of the endothelial phenotype by various noxious stimuli. As a consequence, endothelial cells enable infiltration and retention in the intima of LDLs (low-density lipoproteins), the major cholesterol-carrying lipoprotein in plasma, and contribute to their oxidation. In addition, endothelial cells express adhesion molecules which recruit monocytes. In the vessel wall, monocytes mature into macrophages and take up modified LDLs to the point that their cytoplasm is engorged with cholesterol, generating the so-called foam cells [2], which are crucial to plaque progression in association with smooth muscle cells migrating from the media to the intima. The ongoing inflammatory response sustains and amplifies the process and has a prominent role in driving the complications of the plaque [2].

The treatment of hypercholesterolaemia and hypertension 20–30 years ago was expected to eliminate cardiovascular diseases by the end of the 20th century, but the pathogenic picture has become increasingly complex and such an optimistic prediction needs revision. Thus an understanding of other factors contributing to atherogenesis is necessary in the development of new strategies for prevention and treatment.

Key words: atherosclerosis, endothelial cell, inflammation, magnesium, oxidative stress, thrombosis.
Abbreviations: apoE, apolipoprotein E; EPC, endothelial progenitor cell; GM-CSF, granulocyte/macrophage colony-stimulating factor; HDL, high-density lipoprotein; hsp, heat-shock protein; IL, interleukin; LDL, low-density lipoprotein; MMP, matrix metalloprotease; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; ox-LDL, oxidized LDL; PAI-1, type 1 plasminogen activator inhibitor; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; ROS, reactive oxygen species; TRPM, transient receptor potential melastatin; VCAM, vascular cell adhesion molecule; vW, von Willebrand.
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MAGNESIUM AND CARDIOVASCULAR DISEASES

Environmental factors are fundamental in atherogenesis and, accordingly, changes in lifestyle have yielded excellent results. Intervention on dietary habits is particularly encouraging. There is a general agreement about the inverse correlation between regular consumption of fruit, cereals and vegetables and the risk of atherosclerosis [3]. Edible plant matter contains many beneficial microconstituents, polyphenols, vitamins and minerals. In particular, Mg (magnesium), which is predominantly obtained by eating unprocessed grains and green leafy vegetables, is an essential micronutrient implicated in a large array of regulatory, metabolic and structural activities [4]. The Western diet is relatively deficient in Mg because of low Mg content in water and soil, the processing of many food items, and the preference for calorie-rich and micronutrient-poor diets [5]. This explains why Mg deficiency is relatively common in industrialized countries. Inadequate dietary intake of Mg, however, is not the only cause of Mg deficiency. Mg homoeostasis is very tightly controlled in the intestine and in the renal tubules through a complex network of transporters, some of which have recently been defined at the molecular level [6]. Consequently, Mg deficiency accompanies chronic gastrointestinal and renal diseases, and also complicates diabetes mellitus and therapies with some classes of diuretics, antibiotics or antineoplastic drugs. In addition, it is common in the elderly and in alcoholics [5].

Clinical studies investigating the links between dietary or serum Mg, vascular dysfunction and atherosclerosis have yielded conflicting results. The NHEFS (National Health Epidemiologic Followup Study) found no significant association between serum Mg and the incidence of cardiovascular diseases [7], and similar results were reached in the Framingham Heart Study offspring cohort [8]. However, it should be noted that the measurement of serum Mg, although commonly used in clinical practice, does not fully reflect its homoeostasis [9], Mg being primarily an intracellular cation. In addition, serum Mg should be measured more than once, because of variations in Mg levels depending upon diet, alcohol consumption and physical exercise [10].

In disagreement with the studies described above, the ARIC (Atherosclerosis Risk in Communities) cohort showed an inverse relationship between serum Mg and the development of carotid atherosclerosis [11], and the Paris Prospective Study 2 reported a relationship between low serum Mg and cardiovascular mortality in middle-aged men [12]. In addition, in patients on dialysis, a condition that accelerates atherosclerosis, Mg has a protective role in the progression of the disease [13] and, in renal transplant recipients, hypomagnesaemia is an independent predictor of arterial stiffness [14]. When a link between dietary Mg intake and incidence of cardiovascular diseases was investigated, an inverse association was reported [15,16]. These results are in keeping with the finding that hypomagnesaemia induces a pro-atherogenic lipid profile by decreasing HDLs (high-density lipoproteins) and increasing total serum cholesterol, LDLs and triacylglycerols (triglycerides) [17]. Interestingly, experimental data have demonstrated that Mg deficiency decreases lipoprotein lipase activity, which is implicated in the production of HDL and in the catabolism of triacylglycerol-rich lipoproteins [18], activates HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase), the rate-limiting enzyme in cholesterol synthesis, and LCAT (lecithin:cholesterol acyltransferase), which catalyses the formation of cholesterol esters [19]. Moreover, Mg acts on the lipid profile indirectly, as it modulates insulin action [17]. In particular, Mg deficiency impairs the tyrosine kinase activity of the insulin receptor, an event related to the development of post-receptor insulin resistance [17], which is a well-recognized risk factor for atherosclerosis. To this purpose, it is interesting to recall the recent finding that bittern, a natural MgCl2 solution from the sea widely utilized in Japan to coagulate tofu, can be used as a natural Mg supplement to improve post-prandial hyperlipidaemia in healthy adults [20].

Relevant to atherogenesis is the association of Mg deficiency and oxidative stress [21]. This condition facilitates the oxidation of LDLs, thus potentiating their pro-atherogenic potential, and also promotes inflammation through the activation of the redox-sensitive transcription factor NF-κB (nuclear factor κB), which induces cytokines, growth factors, adhesion molecules and enzymes involved in inflammatory responses [22]. It is noteworthy that an inverse association between Mg intake and the CRP (C-reactive protein) level, a marker of systemic inflammation and a known risk factor for cardiovascular diseases, was reported in different human studies [23-25], and Mg supplementation in overweight individuals led to changes in gene expression and proteomic profile consistent with favourable effects on inflammation [24]. On the basis of human studies, Figure 1 summarizes the mechanisms involved in linking hypomagnesaemia or inadequate Mg intake to atherosclerosis.

The findings from the population studies described above are emphasized by the demonstration that experimentally induced Mg deficiency promotes atherosclerosis in several animal models, mainly through the activation of inflammation and hyperlipaemia [26,27]. Hyperlipaemia and inflammation are considered ‘partners in crime’, since atherosclerosis is an inflammatory disease which is initiated by and progresses in the context of hypercholesterolaemia [28]. Therefore there is sufficient evidence to consider Mg deficiency a multi-faceted contributing factor to atherogenesis.
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Figure 1 How low Mg intake and/or hypomagnesaemia contribute to atherogenesis: a summary from human studies

Human studies indicate that low Mg intake and/or hypomagnesaemia promote inflammation, oxidative stress, insulin resistance and hyperlipaemia, all known risk factors for atherosclerosis.

THE ENDOTHELIUM IN ATHEROSCLEROSIS

Endothelial cells cover the entire inner surface of the blood vessels and are fundamental for the integrity of the vascular wall and for maintaining circulatory functions. Far from being a passive barrier between blood and tissues, the endothelium actively and reactively regulates vascular tone by balancing the synthesis of vasoconstrictors and vasodilators, controls blood fluidity by synthesizing factors that regulate platelet activity and fibrinolysis/coagulation, participates to the inflammatory process by producing cytokines and adhesion molecules that control the ingress and egress of leucocytes and inhibits medial smooth muscle cell growth [29]. Not surprisingly, therefore, endothelial function has been suggested to serve as a barometer for cardiovascular health [30].

In physiological conditions, the endothelium continuously monitors blood-borne and locally generated stimuli by adaptively altering its functional state and by responding to changes in the environment. In response to noxae, such as risk factors for atherosclerosis, the endothelium undergoes functional and structural alterations that jeopardize its protective role and generate a pro-atherogenic phenotype [31]. Damage to the endothelium upsets the balance between vasoconstrictors and vasodilators and triggers various processes that initiate, promote or exacerbate atherosclerosis, such as increased endothelial permeability, reduced NO bioavailability, leucocyte adhesion and synthesis of cytokines (Figure 2). This complex and dynamic process is referred to as endothelial dysfunction. A huge amount of experimental evidence supports the paradigm of endothelial dysfunction as the common link between risk factors and atherosclerotic burden. Endothelial dysfunction is an early event in the atherogenic process and precedes angiographic and ultrasonic evidence of damage to the arterial wall [32]. It also contributes to the later stages of the disease and plays a role in acute coronary syndromes [30]. In clinical practice, numerous methods have been employed to assess endothelial function. Since studies indicate that endothelial function detected non-invasively in the brachial artery correlates with endothelial coronary function, brachial artery ultrasound, which indirectly examines vasodilation in response to stimuli that release NO [33], has emerged as a useful tool for preventive or interventional approaches. By utilizing this technique, it has been demonstrated that interventions proven to reduce cardiovascular risk, such as smoking cessation, lipid-lowering therapy, ACE (angiotensin-converting enzyme) inhibitors and physical exercise, improve endothelial function and decrease cardiovascular risk [32,33]. It is worth highlighting that endothelial function is also significantly correlated to Mg levels and that Mg supplementation results in a significant improvement in endothelial function, associated with an amelioration of exercise duration in patients with coronary artery disease and in diabetic individuals [34–36].

These results in humans are supported by evidence that Mg deficiency has an impact on vascular structure and function in different experimental models. A moderate long-term Mg-deficient diet increases intima-media thickness and stiffness in rats due to increased collagen content and a reduction in the elastin/collagen ratio [37]. In addition, endothelial function is significantly impaired in a model of inherited hypomagnesaemia in mice, as indicated by the decrease in endothelium-dependent vasodilation, which associates with low plasma nitrate levels [38].

Even though studies on cultured cells can only focus on some isolated aspects of the complex events implicated in the development of atherosclerotic plaques, they have produced an extraordinary body of knowledge. It is now time to turn our attention to how endothelial cells, actively involved from the very early to the late stages of atherogenesis, react to culture in low Mg.

MAGNESIUM AND THE ENDOTHELIAL CELL

Mg is the most abundant intracellular bivalent cation, mostly complexed with ATP and other molecules. It is involved in a wide variety of biochemical reactions either by bridging distinct molecules or by functioning as an allosteric modulator through its interaction with negatively charged moieties [4].
Analogously to other risk factors for atherosclerosis, Mg deficiency promotes endothelial dysfunction. Under physiological conditions, the endothelium is a selective barrier to the egress of molecules from the blood. It also maintains the balance between vasodilators and vasoconstrictors and between thrombotic and antithrombotic factors. In addition, healthy endothelial cells do not interact with circulating cells. Low Mg upsets this balance, thus leading to endothelial dysfunction. On the contrary, Mg supplementation in humans and in experimental models, as well as high extracellular Mg on cultured endothelial cells, rescue physiological endothelial function.

Mg homoeostasis in cells is tightly regulated by precise control mechanisms operating at the level of Mg influx and efflux across the plasma membrane, and at the level of intracellular Mg buffering and organelle localization [6]. Mg extrusion occurs against the electrochemical gradient and is mediated through an Na (sodium)-dependent transporter, namely the Na/Mg exchanger driven by the Na gradient, and the Na-independent Mg exchanger [6]. At the present time, these two channels have not been cloned and, consequently, information about their operation and expression are lacking in all tissues. Genetic and electrophysiological approaches have led to the identification of several Mg entry systems [6]. The first molecularly defined components of the mammalian Mg transport machinery are two cation channels of the TRPM (transient receptor potential melastatin) channel family, TRPM6 and TRPM7. They show the unique functional duality of being an ion channel and a kinase, because they possess an active threonine/serine kinase at their C-terminus, which belongs to the atypical family of eukaryotic α-kinases. TRPM7 has a ubiquitous expression pattern, also being expressed in the endothelium, and seems to play a prominent role in intracellular Mg homoeostasis, whereas TRPM6 mainly regulates Mg transport in the kidney and in the gut [6,39]. The presence of functional TRPM7, but not of TRPM6, channels in human endothelial cells has been demonstrated, and a role of TRPM7 in regulating endothelial proliferation and NO synthesis has been described [40].

Recently, the ‘non-imprinted in Prader–Willi/Angelman’ 1 and 2 genes were identified as plasma membrane Mg transporters [6]. The expression of all of these molecules in endothelial cells, however, has not been investigated yet. In addition, Mg is transported in and out of various organelles, mainly mitochondria and Golgi complex. The transporters involved in these pathways have only begun to be identified.

The majority of mammalian cells retain their basal intracellular Mg content unchanged even in the presence of very low extracellular Mg levels [4,6]. Endothelial cells are no exception. Intracellular Mg is not significantly modulated in endothelial cells chronically exposed to low concentrations of the cation [41]. It is noteworthy, however, that Mg deficiency may determine fluctuations in the intracellular content of other ions, namely loss of K (potassium) and accumulation of Ca (calcium) and Na [41]. These alterations might, in part, mediate low Mg effects on endothelial cells.

**Low Mg and endothelial cells: how it all begins**

A relevant issue is to consider how low Mg levels affect endothelial function. There is clear evidence that Mg deficiency, similarly to common cardiovascular risk factors, promotes oxidative stress in various cell types including endothelial cells [42,43]. Free radicals, among which superoxide anions, related ROS (reactive oxygen species) and NO, behave as a double-edged sword. At high concentrations, they are hazardous and damage all major cellular constituents. At moderate concentrations, free radicals play an important role as regulatory mediators in signalling processes, in spite of their ephemeral nature [44,45]. With particular emphasis
on the endothelium, free radicals have a role in regulating signalling cascades that control vasomotor activity under basal conditions. Excessive free radicals, however, activate pro-inflammatory and pro-thrombotic pathways that generate endothelial dysfunction [46].

Culture of endothelial cells in low extracellular Mg rapidly and transiently induces ROS production and reduces intracellular glutathione, a thiol-containing tripeptide which plays an important protective role against cellular oxidative injury by detoxifying free radicals and lipid peroxides [42,43,47]. As a consequence endothelial cells are more susceptible to oxidative injury [43,48]. In parallel, early oxidative DNA damage which resolves within a few hours has also been observed [48]. The primary biochemical source of ROS in the vasculature appears to be the membrane-associated NADPH oxidase enzyme complex, which catalyses the reduction of molecular oxygen using NADPH as an electron donor, generating superoxide [49]. Various cytokines and hormones important in the pathogenesis of vascular diseases up-regulate NADPH oxidase activity and superoxide production. Interestingly, culture in low Mg also induces NADPH oxidase activity in endothelial cells (J.A.M. Maier, unpublished work).

Endothelial cells respond to low-Mg-induced oxidative stress by up-regulating stress proteins, thus activating the typical adaptive response to environmental stresses of different kinds (J.A.M. Maier, unpublished work). It is noteworthy that diverse risk factors for atherosclerosis, among which hypertension, ox-LDL (oxidized LDL) and mechanical stress, evoke stress protein overexpression in endothelial cells [50]. In particular, in endothelial cells cultured in low Mg, hsp (heat-shock protein) 70 is rapidly up-regulated (J.A.M. Maier, unpublished work). Apart from functioning as a chaperone, hsp70 increases endothelial survival and protects these cells from apoptosis by oxidants [51]. Interestingly, in apoE (apolipoprotein E)-knockout mice, which spontaneously develop atherosclerosis, hsp70 is expressed at sites prone to lesion formation before macrophage infiltration has occurred [52], and in human atherosclerotic plaques hsp70 is elevated [50]. In recent years, it became evident that hsps have pro-inflammatory activities and, therefore, contribute to atherogenesis [50].

Stress proteins work in tandem with the ubiquitin–proteasome pathway for protein quality control to prevent the accumulation of damaged proteins [52]. Accordingly, an induction of proteasome activity has been observed in endothelial cells in low Mg [53]. Taken together, these events contribute to reach a new metabolic homeostasis, so that the cells remain viable and metabolically active, although they show clear characteristics of dysfunctional cells. The induction of proteasome activity might also be involved in accelerating the activation of NF-κB in endothelial cells cultured in low Mg (see below).

From oxidative stress to endothelial dysfunction: the increase in permeability and the activation of NF-κB

In endothelial cells, together with the initiation of the stress response, low-Mg-induced oxidants trigger some early events which are crucial in atherogenesis, i.e. the increase in permeability and the activation of the transcription factor NF-κB.

Under normal conditions, the endothelium, with its intercellular tight junctional complexes, functions as a selectively permeable membrane. The excessive generation of free radicals by endothelial cells or other cell types has been implicated in the increased permeability of vascular endothelium to molecules which would not otherwise cross the barrier. Accordingly, antioxidant strategies improve endothelial barrier dysfunction [54]. Low extracellular Mg increases endothelial permeability [55]. Specifically, Mg deficiency enhances LDL transport across the endothelial monolayer [56]. In the first hours this is mainly due to the stimulation of energy-dependent transport, whereas later energy-independent transport also plays a role because of the formation of intercellular gaps. Once LDLs reach the subendothelial space, they are trapped by matrix proteoglycans and become an easy target for oxidant species generated by low-Mg-stressed endothelial cells [57]. It is well known that ox-LDLs are more atherogenic than native LDLs and promote endothelial dysfunction [28,58]. This means that ox-LDL and Mg deficiency might co-operate to alter endothelial function.

Recently, the activation of NF-κB by low extracellular Mg has been demonstrated in endothelial cells [59]. NF-κB is known to play an important role in guarding the delicate balance of the atherosclerotic process as a direct regulator of pro-inflammatory genes and as a regulator of cell survival and proliferation. The potential importance of NF-κB in endothelial cells is further underscored by experiments showing that the endothelium of regions prone to developing atherosclerosis show higher levels of different components of the NF-κB system compared with other less risky regions [60].

In resting cells, NF-κB is inactive and retained in the cytosol because of its association with the inhibitory IκB (inhibitor of NF-κB) proteins. Upon cytokine signalling, innate or adaptive immune responses, or oxidative stress, NF-κB activation is initiated. The phosphorylation of IκBs is an essential step in the activation of the classical NF-κB activation cascade. Phosphorylated IκBs are polyubiquitinated and degraded by the proteasome. Released p50 and p65 NF-κB subunits translocate to the nucleus and bind to NF-κB motifs in target genes, regulating their transcription. Many of NF-κB-regulated genes have been implicated directly or indirectly in atherosclerosis [58,60].

Mg-deficiency-induced oxyradicals activate NF-κB in endothelial cells through the canonical pathway [59].
These findings are corroborated by evidence that Mg supplementation markedly attenuates NF-κB nuclear translocation and protects IκB from degradation in LPS (lipopolysaccharide)-treated endothelial cells [61]. As a result of the activation of NF-κB in the endothelium, low Mg induces the expression of several cytokines, chemokines, growth factors and adhesion molecules, which are critical in atherogenesis.

**IL (interleukin)-1α: a fundamental mediator of low-Mg effects in endothelial cells**

Central in mediating some of low-Mg effects on endothelial cells is IL-1α, a target of NF-κB and an inducer of NF-κB itself. IL-1α is markedly up-regulated early after exposure to low-Mg-containing medium [59]. A founding member of the IL-1 family now comprising 11 members, IL-1α controls pro-inflammatory responses to cell injuries by various stimuli [62]. Animal experiments have clearly demonstrated that IL-1s are major pro-atherosclerotic factors and, accordingly, clinical studies have been initiated with anti-IL-1-targeting antibodies [63].

In addition to binding its surface receptor, IL-1α functions intracellularly, being translocated to the nucleus and influencing transcription [62]. In particular, in endothelial cells endogenous IL-1α must be transported to the nucleus to inhibit cell proliferation, promote cellular senescence and modulate mRNA expression of hundreds of genes, including its own expression, thus generating a positive-feedback loop that amplifies the IL-1 response [64]. It is through the induction of IL-1α that Mg deficiency retards endothelial growth by delaying the transit through the G1- and S-phases of the cell cycle [59,65]. Although endothelial cells are normally quiescent, impaired proliferative capability might become a problem in the case of vascular injury, when it is important to heal the damage to maintain the integrity of the vascular wall. Indeed, endothelial turnover is accelerated by cardiovascular risk factors, including certain lifestyle factors (i.e. lack of exercise, Western diet, pollution and smoking), some diseases (i.e. diabetes and hypertension) and mechanical stress (i.e. catheterization and shear stress) [31]. When this occurs, the damaged cells are removed by the bloodstream and replaced by regenerated endothelium formed mainly by neighbouring cells freed from contact inhibition.

As IL-1α is responsible for endothelial senescence [66], it is relevant that culture in low Mg promotes the acquisition of some features that are typically associated with endothelial senescence [53]. Interestingly, endothelial cells with senescence-associated phenotypes are present in human atherosclerotic lesions and also contribute to atherogenesis because of their altered gene expression [67]. The induction of cell senescence by low Mg, however, is not restricted to endothelial cells [68]. Indeed, inadequate Mg availability accelerates telomere attrition and, consequently, *in vitro* aging of fibroblasts [69].

IL-1α also induces various chemokines and adhesion molecules in vascular endothelial cells through the activation of NF-κB, thus favouring the recruitment, adherence and diapedesis of monocytes. In particular, low Mg stimulates the secretion of IL-8 and RANTES (regulated upon activation, normal T-cell expressed and secreted), chemokines which are overexpressed in human atherosclerotic lesions [70]. IL-8 is critical for the chemotaxis and adhesion of monocytes to the endothelial cells, pivotal events in initiating atherogenesis, and also stimulates monocyte/endothelial interaction by overexpressing VCAM (vascular cell adhesion molecule)-1 on the endothelial surface [53,65]. VCAM, up-regulated in the arterial intima of human atherosclerotic plaques, functions as both a scaffold for leucocyte migration and a trigger of endothelial signalling through NADPH-oxidase-generated oxidant species [71], thus exacerbating the direct effects of low Mg on NADPH oxidase activity. Free radicals induce signals for the opening of intercellular passageways through which monocytes migrate [44,45]. In addition, the secretion of GM-CSF (granulocyte/macrophage colony-stimulating factor) is markedly increased in Mg-deficient endothelial cells [65]. GM-CSF regulates the differentiation of cells of macrophage lineage, an event which takes place once monocytes have reached the intima.

All these findings point to Mg deficiency as a potential contributor to the accumulation of monocytes/macrophages in the arterial wall during the early stages of atherogenesis.

An inadequate availability of Mg also stimulates the secretion of PDGF (platelet-derived growth factor)-BB by endothelial cells [59,72]. To this purpose, it is worth noting that PDGF-BB is a direct target of NF-κB, an effect that can be potentiated by the presence of IL-1α, which is known to stimulate PDGF production [73]. However, it is also possible that PDGF induction results from monocyte–endothelial interactions and is mediated by adhesion molecules [74]. A huge amount of experimental evidence is available about the contribution of PDGF to atherogenesis [58]. First, PDGF expression is increased in human atherosclerotic arteries [75]. Secondly, PDGF, which is chemotactic and mitogenic for smooth muscle cells, co-operates with other cytokines and growth factors in driving the accumulation of these cells in the intima, where they change phenotype, lose myofilaments, enrich their content of Golgi and rough
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**Figure 3** How low extracellular Mg affects cultured human endothelial cells: implications in the early phases of atherogenesis

In vitro studies on endothelial cells have demonstrated that low Mg promotes the acquisition of a pro-inflammatory pro-atherogenic phenotype in endothelial cells. Culture in low Mg leads to the production of free radicals which: (i) induce the expression of hsp's involved in protecting the cells from death; (ii) increase endothelial permeability, an event which facilitates LDL accumulation in the subendothelial space; and (iii) activate NF-κB, thus up-regulating adhesion molecules, cytokines, chemokines and growth factors. In addition, the increased activity of the proteasome might contribute to the activation of NF-κB. The up-regulation of IL-1α has a role in inducing endothelial senescence.

Endothelial cells are pivotal in maintaining the balance between anti- and pro-thrombotic factors. Under normal circumstances, endothelial cells actively prevent the fibrous cap from breaking down, ensuring the stability of the plaque. However, in the presence of low Mg, the balance is disrupted, leading to the up-regulation of MMPs (matrix metalloproteinases), which degrade collagen fibrils and cause the loss of the fibrous cap. This process is facilitated by the activation of NF-κB, which up-regulates gene expression of MMPs.

**Towards the end: low Mg as an inducer of proteases and pro-thrombotic factors**

Maintenance of the fibrous cap reflects the balance between matrix production and degradation. An active role of the endothelium in the remodelling of extracellular matrix and, therefore, its potential to contribute to plaque instability has been described. In atherosclerotic plaques, the expression of MMPs (matrix metalloproteinases), which degrade collagen fibrils leading to the loss of fibrous cap integrity, is increased. In particular, a marked up-regulation of MMP-9 in unstable carotid plaques has been demonstrated and genetic variations within the MMP-2 promoter region have been associated with cap thickness, with consequent influences on plaque vulnerability. The expression of these proteases is normally tightly regulated at the transcriptional level. A second level of control is represented by the requirement of the extracellular activation of the latent pro-enzyme. A third level of control on MMP activity involves the irreversible binding of these proteases to their specific tissue inhibitors (TIMP, tissue inhibitor of metalloproteinases).

In endothelial cells cultured in Mg-deficient medium, a marked increase in the total amounts and activity of MMP-2 and MMP-9 has been reported. Accordingly, MMP-2 and MMP-9 determine the structural alterations of the vascular wall in Mg-deficient rats. An NF-κB-binding site is present in the promoter of the MMP-9 gene. It is therefore possible that low Mg availability might directly increase MMP-9 expression via NF-κB in the beginning and sustain it through IL-1α later. The mechanisms underlying MMP-2 induction by low Mg are still a matter of investigation. Regarding the activation of the pro-enzymes, free radicals might be involved. Indeed, oxyradicals are known to react with thiol groups, including those preserving MMP latency, and therefore activate MMPs. In particular, in cultured smooth muscle cells exposed to mechanical stress, free radicals derived from NADPH oxidase increase the activity of MMP-2 and MMP-9.

It is noteworthy that, in Mg-deficient endothelial cells, MMP-2 and MMP-9 activity overrides the inhibitory effect of TIMP-2, which is also induced, probably as an attempt to counterbalance the detrimental effects of the proteases.

Endothelial cells are pivotal in maintaining the balance between anti- and pro-thrombotic factors. Under normal circumstances, endothelial cells actively prevent the formation of the fibrous plaque. In the presence of low Mg, the balance is disrupted, leading to the up-regulation of MMPs and the degradation of the fibrous cap.
thrombosis by synthesizing molecules that block platelet adhesion and aggregation, inhibit coagulation and lyse the clots [29]. Healthy endothelium produces prostacyclin and NO, which are co-released and form a particular partnership to impede platelet adhesion [83]. Culture in low Mg increases the endothelial synthesis of prostacyclin [84], probably as the result of the induction of COX-2 (cyclo-oxygenase-2) by free radicals and IL-1α. It is interesting to note that this enzyme is expressed and contributes to the increase in prostacyclins in patients with atherosclerosis [85]. Low Mg also seems to induce NO both in a model of Mg deficiency in rodents [86] and in cultured endothelial cells [87], and this might be explained within the framework of the inflammatory response activated by low Mg. However, the superoxide radical produced by NADPH oxidase under Mg-deficient conditions reacts very rapidly with NO, thus reducing NO bioactivity and generating peroxynitrite, another reactive species with damaging effects on macromolecules.

Low Mg also affects endothelial fibrinolytic activity. Indeed, PAI-1 (type 1 plasminogen activator inhibitor), which inhibits tissue plasminogen activator thereby preventing fibrinolysis, is up-regulated in endothelial cells in low Mg [65] and in the aortas of hypomagnesaemic mice [38]. It is noteworthy that PAI-1 is induced in inflammation and is overexpressed in senescent endothelial cells [88], two features strictly associated with Mg deficiency. High plasma levels of PAI-1 are important in the pathogenesis of thrombotic disease and an up-regulation of PAI-1 has been demonstrated in human atherosclerotic lesions [89]. Accordingly, deletion of the PAI-1 gene completely reverses the prothrombotic tendency of apoE-knockout mice despite the persistence of excessive hypercholesterolaemia [90]. Figure 4 summarizes these results and links them to their potential role in the complications of atherosclerotic plaques.

In conclusion, Mg deficiency plays a role also in events involved in the late phases of atherosclerosis by having an impact on the endothelial synthesis of proteases and pro-thrombotic factors.

**Elevated extracellular Mg and endothelial cells**

All of the data available on cultured endothelial cells highlight that low extracellular Mg markedly alters endothelial function. The obvious question is whether endothelial dysfunction is reversible and the answer is yes. After culture in low Mg, the re-addition of the cation to the medium to reach the physiological concentration rescues the normal proliferation rate and the physiological activities of endothelial cells, thus indicating that no permanent modifications occur with Mg deprivation [65].

To support findings showing that Mg supplementation is beneficial in patients with coronary artery disease and diabetes [34–36], evidence has been provided that culture of endothelial cells in high Mg increases NO production [91], prevents NF-κB activation and, therefore, the induction of cytokines and chemokines [61], decreases MMP-9 activity [92], and up-regulates proteolysis of ultra-large vW (von Willebrand) factor [93], thus
inhibiting platelet adhesion (Figure 2). Accordingly, in rats, the infusion of Mg blocks the formation of thrombi after vascular injury by reducing platelet aggregation and prolonging blood clotting time [94].

CONCLUSIONS AND FUTURE PERSPECTIVES

Studies on endothelial cells teach us that low Mg promotes the acquisition of a pro-atherosclerotic and pro-thrombotic phenotype by having an impact on events involved in the initiation, progression and complications of the plaque. In spite of the fact that some clinical studies found no correlation between serum Mg and cardiovascular disease [7,8], in vitro and in vivo studies have clearly demonstrated that Mg deficiency not only affects endothelial cells, but also smooth muscle cells [95], thus highlighting the notion that an inadequate availability of the cation targets two principal players in atherogenesis.

New fascinating aspects of endothelial function are emerging from research on EPCs (endothelial progenitor cells) [96]. Recent insights indicate that the injured endothelium can be regenerated by circulating bone-marrow-derived EPCs, which accelerate re-endothelialization and limit atherosclerotic lesion formation. It is also clear that risk factors for atherosclerosis, among which age, hypertension, smoking, hypercholesterolaemia and diabetes, reduce the number and functional activity of these circulating EPCs, thus limiting the regenerative capacity and contributing to atherogenesis and atherosclerotic disease progression. At the moment it is not known whether and how Mg deficiency affects the number and the functions of EPCs. It is likely that broadening our knowledge in this novel field, together with deepening our understanding on the effects of Mg and its transporters on the cells implicated in atherogenesis, will rejuvenate research in the field and, hopefully, open new avenues to prevent and treat vascular diseases.

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