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MAGNESIUM AND ENDOTHELIAL FUNCTION: COMPARATIVE STUDIES IN MACRO AND MICROVASCULAR ENDOTHELIAL CELLS

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"Non sempre le cose sono quello che sembrano. La prima impressione inganna molti.
L'intelligenza di pochi percepisce quello che è stato accuratamente nascosto."
Fedro

La cosa più bella con cui possiamo entrare in contatto è il mistero. E' la sorgente di tutta la vera arte e di tutta la vera scienza.
Albert Einstein

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1. LITERATURE REVIEW

A. MAGNESIUM

Magnesium (Mg), the most abundant intracellular divalent cation in living cells, with a molecular weight of 24.305, plays a vital role in many cellular processes. It is the fourth most abundant cation in the organism $Ca^{2+}>K^{+}>Na^{+}>Mg^{2+}$ and the second in the intracellular fluid after K^{+}. The abundance of Mg within mammalian cells is consistent with its relevant role in regulating tissue and cell functions.

Almost every biological process requires Mg. It has been suggested that free intracellular Mg ([Mg]i) may act as a second messenger which modulates cellular functions, affecting not only channel activities and effectors but also the secretion of various biologically active substances such as hormones and cell signaling mediators (Laurant and Touyz 2000; Takaya et al., 2000).

1. BIOCHEMICAL AND BIOLOGICAL ROLE OF MAGNESIUM

1.1 Biochemical role

Mg is essential for a number of metabolic activities since it is associated with a number of enzymes which control carbohydrate, lipid, protein and electrolyte metabolism. In the cytosol, the majority of Mg is bound to ATP and other phosphonucleotides, and to many enzymes.

- Mg and channel activity. Mg is an important membrane stabilizing agent and a physiologic calcium (Ca) blocker. Because of its high intracellular concentration, Mg plays an important role as an ion pump and channel regulator. The relation between Mg and channel activity goes along with the regulation of Ca, an important second messenger. It has been shown that Mg inhibits Na/Ca exchange, influences the ATPases as well as the voltage operated channels (VOC) (Bara et al., 1989; Iseri and French, 1984 and Laurant and Touyz, 2000). Many works have pointed to the role of Mg in regulating some receptors among which muscaranics, endomorphics and β-receptor N-methyl-D-aspartate (NMDA) where Mg acts by modifying the membrane potential (Begon et al., 2002; Crosby et al., 2000).
- Mg and plasma membrane. Mg forms complexs with phospholipids (phosphatidilserine) of the plasma membrane. This complex increases membrane stability and modifies the transmembrane influx of electrolytes. Cytosolic free Mg is constant in physiological conditions. It has been proposed that the plasma membrane may posses a sensor or some very specific uptake/release mechanism to maintain these values constant (Romani and Scarpa, 2000; Such and Hille, 2007).

- Mg and enzymes. Many enzymes appear to require and to be regulated by Mg for their activity but the exact number appears to be uncertain (Gunther 2008). All the enzymes utilizing ATP require Mg for substrate formation. Intracellular concentration of free Mg acts as an allosteric activator of enzyme action including critical enzyme system such as ATPases, guanosine triphosphatase, phospholipase C, adenylate cyclase and guanylate cyclase and others. The ionic transport pumps such as Na/K-ATPase, Ca-ATPase and H-ATPase require Mg for their activity. Therefore, transport of ions and cations across the plasma membrane may also require the presence of Mg (Dorup and Clausen, 1993; Garfinkel L and Garfinkel D, 1985).
- Mg and phosphate groups. Mg reacts with phosphate groups; hence an interaction with every biological phophate-containing molecule is expected and explains in part its biochemical properties. Mg is necessary for triphosphate nucleotide synthesis, phosphorylation and redox reactions in the mitochondria. Mg is cofactor for adenosine triphosphate (ATP) and it is also linked to ATP, forming the Mg-ATP complexes, and only in this complex the ATPases can hydrolyze ATP.
- Mg and nucleotides. Mg is essential for every enzymatic reaction which has nucleotides as substrates. The mechanism by which it excerts this function is explained by its link to phosphate groups. It is also involved in transcriptional, nucleic acid polimerization and translational processes (Vernon, 1988). Mg has been shown to be related to the stabilization and degradation of DNA and RNA and to regulate the synthesis of cyclic adenosine monophosphate (cAMP), an important intracellular second messenger (Laurant and Touyz, 2000; Takaya et al., 2000).

1.2 Biological role

Mg biological role is extremely versatile as it can serve structural functions (e.g. fluidity and stability of phospholipid bilayers, protein tertiary or quaternary structures and DNA double helices) as well as dynamic functions (e.g. cofactor or allosteric modulator of enzyme activities). It is expected that the multiple biochemical function of Mg in the organism and hence its status may influence several biological events among which hormone secretion. The secretion of cathecolamine, acetylcholine, insulin, histamine, serotonin, parathormone can be modified by extracellular Mg concentration ($[Mg]_e$). Mg may therefore influence endocrine, nervous, cardiovascular and immune system.

In the central nervous system $[Mg]_e$ stabilizes the nervous fiber membrane becoming less excitable, increases the affinity of the agonists dopaminergic for the D2 receptors, the agonists α and β, and blocks Mg central receptors such as NMDA (Begon et al., 2002; Crosby et al., 2000; Jeong et al., 2006; Mayer et al., 1984; Ulugol et al., 2002). As nervous and immune system interact, the roles of neuromediators and their consequences are related. In the immune system Mg can exert an important function in blocking not only NMDA receptors but also the activation of nuclear factor-kappa (NF-kB) (Mazur et al., 2007).

In the heart it has been shown that [Mg]i modulates Ca efflux from the sarcoplasmic reticulum and the cell, activates the Na/K ATPase pump and reduces indirectly intracellular Ca and muscular contraction (Berthelot et al., 2004). This coupling between ionic currents and intracellular Ca homeostasis, together with the increase of membrane potential and the decrease of the excitability of the muscular fiber by [Mg]e, underlies the regulation of cardiac cell function (Michailova and McCulloch, 2008).

In the vascular system, Mg influences vascular tone by regulating endothelial and vascular smooth muscle cell (VSMC) functions. Mg stimulates prostacyclin production and nitric oxide (NO) formation and promotes endothelium-dependent and endothelium-independent vasodilatation. Isolated vessels exposed to reduced levels of Mg display a transient vasorelaxation followed by sustained constriction. In the presence of endothelial damage, low Mg induces a sustained contraction without the transient vasorelaxation phase, suggesting that Mg could have a dual effect in the regulationof vascular reactivity depending on the integrity of the endothelium (Gold ME et al., 1990; Touyz RM. 2007).

Mg also modulates vascular tone and reactivity by altering responses to vasoconstrictor and vasodilator agents. Increased $[Mg]_e$ blunts vasoconstrictor and vasorelaxant properties of vasoactive agents (Ko EA et al. 2005; Soltani N et al., 2005). These effects may be related to altered binding of agonists to their specific cell membrane receptors and/or to production of vasoactive agents such as endothelin-1 (ET-1), angiotensin II (ANG II) and prostacyclin (PGI2) (Laurant P and Berthelot A, 1996; Satake K et al., 2004).

Another possible mechanism whereby Mg modulates vascular function is via its antioxidant, anti-inflammatory, and growth regulatory properties (Touyz RM et al., 2002; Touyz RM and Schiffrin EL, 2004). Vascular cells are a rich source of reactive oxygen species, which directly alter VSMC growth. Mg has antioxidant/anti-inflammatory properties that attenuate damaging actions of oxidative stress and inflammation in vasculature, thereby preventing vascular injury

(Mazur et al., 2007;Weglicki WB et al.1992, Weglicki WB et al, 1996). These effects may be important in hypertension, where generation of reactive oxygen species in increases and [Mg]i is reduced (Taniyama Y and Griendling KK, 2005).

2. MAGNESIUM DISTRIBUTION

2.1 Intracellular distribution

Mg is contained within all intracellular compartments. Total cellular Mg concentration can vary from 5 to 20 mM, depending on the type of tissue studied. [Mg]i is predominantly complexed to organic molecules such as ATP (80-90%), cell and nuclear membrane-associates proteins, DNA, RNA, enzymes, proteins and citrates. Mg is distributed differently within the organelles (~10mM), especially mitochondria, 2-5 mM in complex with ATP in the cytosol, and trace amounts complexed with enzymes (Berthelot et al., 2004; Romani and Scarpa, 2000; Romani AM, 2007). Athough Mg is abundant within the cell, only a small fraction of [Mg]i (1% to 3%) exists as the free ionized form of Mg, which has a closely regulated concentration of 0.5 to 1 mM (de Rouffignac and Quamme, 1994).

2.2 Body distribution

The recommended dietary allowance of Mg is 300 mg/day for adult non-pregnant woman and 350 mg/day for adult man. The normal adult body Mg content is about 25g which corresponds to one mole or 2000 mEq and its distribution is approximately equally divided between the skeleton and soft tissues. A large proportion (about one third) of skeletal Mg resides on the surface of bone. Because this fraction is exchangeable and because the skeletal Mg level falls during Mg depletion, it is hypothesized that this component serves as a reservoir to maintain the extracellular Mg concentration. $[Mg]_e$ accounts for only 1% of total body magnesium (serum and intestinal fluid) and its concentration is equal to the one in the vascular compartment. In serum, Mg is present in three fractions; protein bound (27%), ionized (65%), and complexed with anions such as phosphate, bicarbonate, and citrate (8%) (Speich et al.,1981). In the plasma, Mg concentration is in the range of 0.9-1 mM of which around 55% is ionized or free, 15% is complexed to anions, and the rest (almost 30%) is bound to proteins (mainly albumin) (Rude,1998).

Mg is widely distributed in foods and hard water. Generally, the daily-required minimum level of this mineral can be achieved with a varied diet. Refining or processing of food may deplete

Mg content by nearly 85%. Furthemore, cooking - especially boiling Mg rich foods -will result in a significant loss of Mg (Fawcett et al., 1999). It has been observed that chlorophyll of green vegetables is a good source of Mg (U.S. Department of agriculture, 2003). High sources of Mg include spinach, soybeans, unrefined bran-grains, wheat, oatmeal, nuts, brown rice, black-eyed peas and cereals. Drinking water can be an important source of Mg. Tap water can be a source of Mg, but the amount varies according to the water supply. Hard water may content up to 30 mg/l of Mg.

In some studies dietary Mg was not correlated to serum Mg because renal Mg handling affects total body serum Mg more than a normal dietary Mg intake. Nevertheless, an inverse association between dietary Mg and coronary heart disease incidence in humans suggests that high intake of foods rich in Mg may provide protection against coronary heart disease (Liao F. et al., 1998; Shechter M., 2010).

3. MAGNESIUM HOMEOSTASIS

Mg homeostasis in humans primarily depends on the balance between intestinal uptake and renal excretion (Figure1). Three organs determine the plasma Mg level, namely, the intestine by which Mg is taken up from the food, bones, which store and release Mg, and the kidney, which determines the excretion of Mg.

- In the intestine, physiologic studies indicate two different transport systems for Mg absorption: i) a saturable, active transcellular transport mediated by carriers and ii) a non saturable, passive paracellular pathway (Figure 1A and 1B).

The saturable transcellular uptake consists of an apical entry into the epithelial cell through a specific ion channels and a not yet defined basolateral extrusion mechanism which probably couples Mg export to sodium influx. Recently two transient receptor potential (TRP) channel family members, TRPM6 and TRPM7, were shown to be involved in the regulation of Mg homeostastis. They were identified by chance in the quest for elucidating the molecular bases of hereditary inborn errors of Mg handling (Schlingmann KP et al. 2002; Schlingmann KP and Konrad M, 2004).

The major driving force for the passive diffusion of intestinal Mg is the electrochemical gradient across the epithelium. Absorption by this process is expected to vary with the luminal Mg concentration and does not demonstrate saturation kinetics (Brannan et al., 1976; Fine et al., 1991; Schlingmann et al., 2004). More detailed studies have confirmed that the concentration of

ionic Mg on the luminal side of the absorption site is the major factor in the regulation of the amounts of Mg absorbed over a given time interval (Hardwick et al., 1991).

With a normal dietary Mg intake of approximately 300-350 mg/day, fractional intestinal absorption is 30-50% (Schwartz et al. 1984). This variation may be due to the presence of other nutrients that interact with Mg in the gut. A high dietary content of fibers, phytates, oxalates, and phosphates reduces Mg absorption by binding the cation. Also protein-rich diets of <30 g/day hinder Mg absorpion.

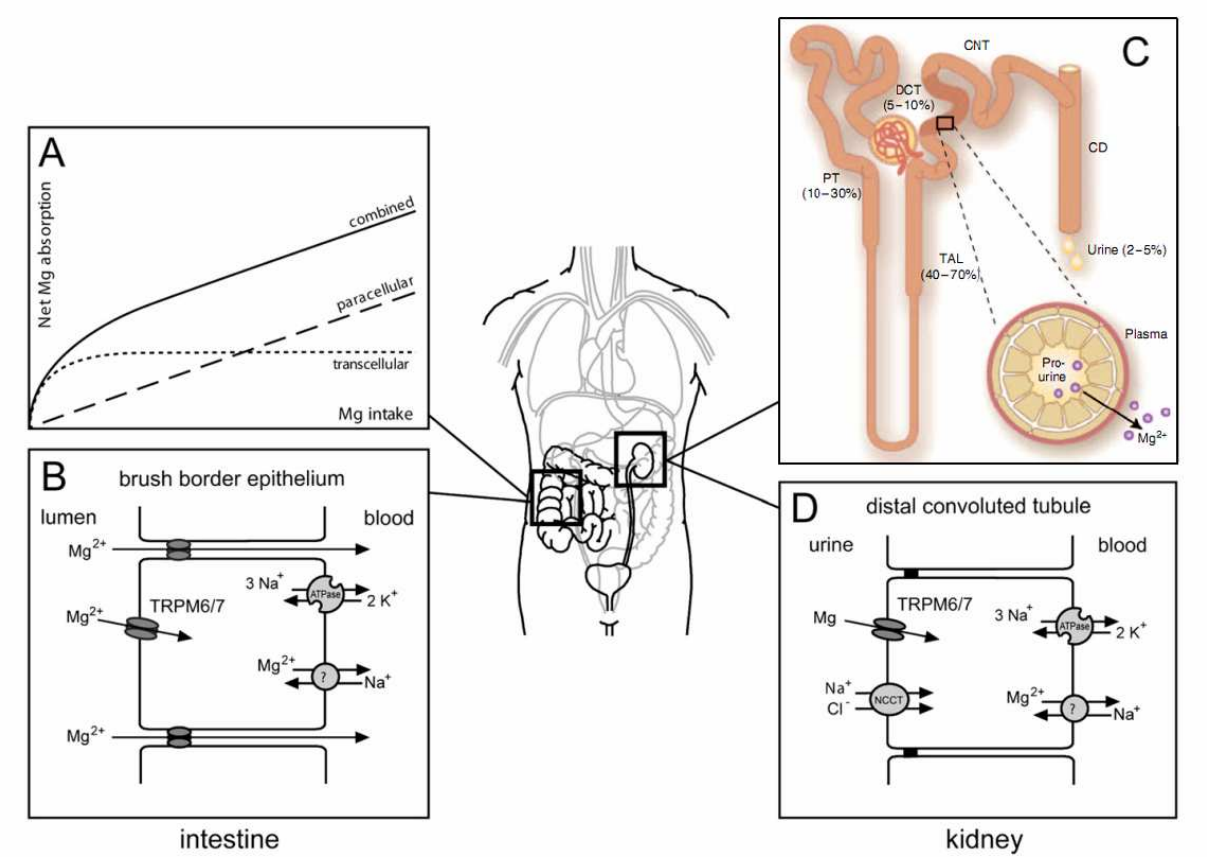

Figure 1: **Epithelial Mg transport in the intestine and kidney**.

(A) Intestinal absorption follows a curvilinear kinetic resulting from two transporter mechanisms.

(B) Intestinal absorption results from two transport mechanisms: i) a saturable transcellular transport by TRPM6/7 and ii) paracellular passive transport.

(C) Mg transport along the nephron.

(D) Mg apical entry into the distal convoluted tubule (DCT) cells through: i) probably TRPM6/TRPM7 heterodimers and ii) a basolateral extrusion step.

➢ The highest percentage (50-60%) of total body Mg is stored in the mineral phase of the skeleton. The majority of Mg in bones is incorporated into the hydroxyapatite crystal. One third is surface limited and appears to serve as a reservoir for maintaining extracellular Mg concentration.

It is hypothesized that bone serves as a buffer for plasma Mg. At this point, little is known about the mechanisms by which Mg is stored in bone by osteoblasts and released by osteoclasts.

- The kidneys are involved in the regulation and fine-tuning of the final Mg concentration in plasma. In the kidney the regulation of Mg balance is due to changes in renal excretion-reabsorption, largely influenced by extracellular Mg concentration. There is a threshold of filtered Mg below which Mg is conserved and above which Mg is totally excreted (Rude and Ryzen, 1986). This threshold is close to the normal plasma Mg concentration. Excessive Mg, either dietary or parenterally administered, is almost totally excreted. In contrast, at the time of Mg deprivation, the kidney avidly conserves Mg; less that 12-24 mg is excreted per day. Dietary factors may also affect renal Mg excretion. High sodium, calcium, alcohol, protein-rich diets as well as caffeine may increase renal Mg excretion.

Each day, ~2500 mg of Mg are filtered by the glomeruli and 90-95% is reabsorbed along the nephrons (Figure 1C) (Dai LJ et al., 2001). The highest level of reabsorption occurs in the proximal tubules (10-30%) and the cortical thick ascending limbs of Henle's loop (TAL) in a passive paracellular manner (40-70%) driven by lumen-positive electrochemical gradient (Mandon et al., 1993; Cole and Quamme, 2000). The mechanisms that manage Mg transport in the proximal tubules are unknown, whereas in the TAL, Mg reabsorption is facilitated by a specific divalent cation permeant pore formed by proteins of the claudin family of tight junction proteins. In 1999 Simon and coworkers discovered the first component of the paracellular pathway called "paracellin-1" or "claudin-16", a specific pore-forming protein (Simon DB et al., 1999). Recently, Konrad and colleagues have reported the involvement of a second member of the claudin family called "claudin-19", another tight junction protein (Konrad M et al., 2006). Mutations of claudin-16 and claudin-19 are causative for familial hypomagnesemia with hypercalciuria and nephrocalcinosis (Konrad M et al., 2006).

The final 5-10% of the filtered load is reabsorbed by the distal convoluted tubule (DCT) (Figure 1D), which consists of two subsegments, namely, DCT1 and DCT2. The DCT1 segment determines the final Mg concentration, as the more distal parts of the tubule are largely impermeable to Mg. In DCT1, Mg reabsorption occurs in an active transcellular manner. The molecular component directly involved in the transcellular pathway remained unknown until the discovery of TRPM6 as a possible candidate of the apical ephitelial Mg channel. TRPM6 is localized on the luminal membrane of DCT cells and the brush-border membrane of the intestine as mentioned above. The Mg concentration in pro-urine is likely around 1.1 mmol/l, whereas the free [Mg]i has been estimated to be 0.5-1.0 mmol/l. The luminal membrane potential in DCT is

approximately –70mV, favouring luminal Mg influx. Thus, the movement of Mg into the DCT cell through TRPM6 seems mainly driven by the electrical gradient.

B. MAGNESIUM DEFICIENCY

1. MAGNESIUM DEFICIENCY IN HUMANS

Mg deficiency and hypomagnesemia are highly prevalent in industrialized countries (Nadler and Rude, 1995). Hypomagnesemia is consequent to negative Mg balance that results from chronic low Mg intake, increased gastrointestinal or renal losses, a shift of Mg from extracellular to intracellular compartments, and genetic factors (Davenport et al.,1990; Dunn and Walser, 1966; Henrotte et al.,1990 Naderi and Reilly, 2008; Quamme, 1993; Rude et al., 1999).

Hypomagnesemia occurs in patients with diabetes mellitus type II, metabolic syndrome, HIV, in alcoholists, in individuals receiving some classes of diuretics and chemotherapeutics such as cisplatin and cetuximab (Deheinzelin D. et al., 2000) (Table1). Hypomagnesemia has been reported in 63% of intensive care patients and up to 45% of patients with acute myocardial infarction and it is associated with increased mortality. Hypomagnesemia is frequently an acquired disorder, only in rare cases it has an underlying hereditary etiology that can cause a progressive dilated cardiomyopathy and heart failure (Henrotte et al., 1990; Schlingmann et al., 2004). Henrotte studied inhereted hypomagnesemia in populations and found a polygenic influence and probably polymorphisms in genes responsible for Mg homeostasis.

I.	Low Mg intake: • Food processing • Wasted soils • High phytate diet • Low oxalate diet
II.	Gastrointestinal losses: • Malabsorption syndrome • Chronic diarrhea • Intestinal resection • Primary infantile hypomagnesemia
III.	Renal disease: • Post obstructive nephopathy • Post renal transplantation • Dialysis • Diuretic phase of acute renal failure
IV.	Shift of Mg from extracellular fluid into cells or bone
V.	Others (alcoholism, drugs, diabetes)

Table1 : Etiology of Mg deficiency and hypomagnesemia.

1.1 Diagnosis of Mg deficiency

Serum or plasma concentration is the most utilized clinically available test for assessing Mg status (Saris et al., 2000). Total serum Mg concentration, of which 65-75% is diffusible, is usually 1.7-2.2 mg/dl (1.5-1.9 mEq/l) and appears to be tightly held within this range. A serum concentration of less than 1.7mg/dl usually indicates some degree of Mg depletion. The measurement of serum Mg concentration, however, may not reflect the true total body Mg content. Low intracellular magnesium has been documented in patients with serum levels above 1.7 mg/dl. Intracellular levels in muscle, red blood cells, lymphocyte and bone Mg can be measured and appear to better assess body Mg status but they are not readily available for clinical use (Rude RK et al., 1991).

Physiological test such as metabolic balance studies, urinary excretion of Mg and Mg loading test are of big value in determining Mg status and excretion. Urine excretion of Mg may reflect intestinal absorption and may determine whether Mg wasting occurrs by renal route (Fawcett et al., 1999). The Mg loading test has been used for many years and seems to be the gold standard of Mg status (Arnaud 2008). This test consists in evaluating Mg retention after parenteral or oral administration of Mg.

1.2 Clinical manifestation of hypomagnesemia

Generally, individuals with moderate hypomagnesemia or Mg deficiency do not present any symptoms. Signs and symptoms are usually seen when Mg decrease to at least 0.5 mM (Swaminathan, 2003). The symptoms of Mg deficiency (Table 2) are in part related to electrolytes disturbances secondary to Mg deficit, such as hypokalemia, hypocalcemia and metabolic alkalosis (Konrad and Weber, 2003; Schlingmann et al., 2004). In humans and experimental animals, early symptoms of hypomagnesemia are nonspecific and may include lethargy and weakness. However, as hypomagnesemia progress, increase neuromuscolar excitability (tremors, capopedal spasm, muscle cramps, tetany and generalized seizures, convulsions), anorexia, decrease sleep time, hypokalemia and hypocalcemia are observed (Naderi and Reilly, 2008; Chollet et al., 2001; Kruse et al., 1979). Clinical hypomagnesemia has been associated with a higher incidence of arrhythmias, vasospasm, sudden death with congestive heart failure, and acute myocardial infarction (Kramer et al., 2009).

Table 2: Clinical manifestation of hypomagnesemia and Mg deficiency.

a. ELECTROLYTE DISTURBANCES	Hypokalemia Hypocalcemia Hypophosphatemia Hyponatremia
b. HORMONAL DISTURBANCES	Hypoinsulinemia, increase insulin resistence Enhanced angiotensin II action Impairment of Vitamin D matabolism
c. SYSTEMIC DISTURBANCES	Inflammation (immune system) Nervous system Glucose metabolism Cardiovascular manifestations Metabolic syndrome Osteoporosis

Patients suffering from severe hypomagnesemia are often supplemented with Mg. A high dose of Mg, however, can have adverse effects such as diarrhea and abdominal cramping. Furthemore, Mg salts are often utilized in case of severe asthma attack and to treat pre-eclampsia in pregnant women (Silverman R et al., 2002; Gabbe SG, 1996).

a. Electrolyte disturbances

Mg deficiency has been associated with electrolyte disturbances (secondary to the mineral deficit), including hypokalemia, hypophosphatemia and hypocalcemia (Konrad and Weber, 2003; Nadler and Rude, 1995; Schlingmann et al., 2004).

- Hypokalemia. Hypokalemia is a common event occuring in 40 to 60% of hypomagnesemic patients (Agus 1999). Primary disturbances in Mg balance produce secondary K depletion (Rude 1998). Thus low intracellular K in Mg deficiency appears to result from an inability of the cell to maintain the normally high intracellular concentration of K. Probably this is a result of an increase in membrane permeability to K and/or inhibition of Na/K-ATPase. Finally, this leads to an increase in K secretion in the loop of Henle and in the collecting duct (Berkelhammer and Bear, 1985; Solomon, 1987). A decrease in [Mg]i caused by Mg deficiency releases the Mg-mediated inhibition of Renal Outer Medullary Potassium (ROMK) channels and increase K secretion. Mg deficiency alone, however, does not necessarily cause hypokalemia. Increase of distal Na delivery or elevated aldosterone levels may be required for exacerbating K wasting in Mg deficiecy (Huang and Kuo, 2007).

The arrhythmias which occur in Mg deficiency may be atrial or ventricular and the arrhythmogenic effect may be related to Mg effect in maintaining proper concentrations of intracellular K (see below). Mg is necessary for Na/K ATPase which is responsible for active transport of K intracellularly during the action potential duration. Mg is also involved in regulating K influx through different K channels. A deficiency of myocardial Mg can lead to a decrease of intracellular K due to a less efficient Na/K-ATPase system and also by the loss of inward rectification (Matsuda H., 1991; White RE et al.,1989).

- Hypocalcemia. Hypocalcemia is another common manifestation of moderate to severe Mg deficiency in humans as well as most other species (Shils 1969; Suh et al.,1971). Hypomagnesemia might worsen hypocalcemia via impairments of vitamin D metabolism and PTH action. The majority Mg-deficient hypocalcemic patients have low or inappropriately normal PTH levels. Hypocalcemia stimulates the secretion of PTH, which would, in turn, restablish a normal plasma Ca level except in severe situations (Suh et al.,1971). In chronic severe hypomagnesemia, failure to correct hypocalcemia could be related to the following mechanisms involving parathyroids: a) diminished responsiveness of endorgans to PTH, b) excessive inactivation of circulating PTH, c) impaired synthesis or diminished secretion of hormone by the parathyroid glands (Kanazawa et al., 2007).

- Hypophosphatemia. Mg depletion can also lead to phoshaturia and decreased intracellular phosphate content (Berkelhammer and Bear 1985).

b. Hormonal disturbance

In general, hypomagnesemia promotes insulin resistance and PTH release, decreases the activity of 1-α-hydroxylase and enhances angiotensin II (ANG II) action, thus affecting K, Ca and Na balance.

- Hypoinsulinemia/increased insulin resistance. Some evidence suggests that Mg may play a role in insulin-mediated glucose uptake (Paolisso et al., 1988). Low Mg status can impair insulin release and induces insulin resistance (Hans et al., 2003). Insulin itself is an important regulatory factor of intracellular Mg (Khan et al., 1999; Rodriguez-Moran and Guerrero-Romero, 2003). In diabetes, low Mg contributes to maintaining continuous high blood glucose levels. The mechanisms by which Mg deficiency can alter insulin action are not clear. However, it appears that Mg does not interfere with insulin binding to its receptor.

Other evidence shows that Mg deficiency can lead to changes of intracellular Ca levels that have been reported to contribute to insulin resistance. It is also possible that Mg deficiency can directly alter activity of enzymes involved in glucose metabolism (Nadler et al., 1993).

- Enhanced action of angiotensin II (ANGII). Studies in isolated vessels indicate that a reduction of Mg can markedly enhance the response to ANGII. Furthemore, Rude and coworkers have shown that dietary induced Mg deficiency in healthy humans can enhance ANGII-induced increases in blood pressure (Rude RK et al.,1989). Elevated levels of ANGII in Mg deficiency may be due to increased angiotensinogen expression and/or increased renin activity and not due to increased conversion of ANGI to ANGII because no increment in serum angiotensin-converting enzyme (ACE) activity was observed (Spagna et al., 2006). Increased ANGII secretion also induces secretion of aldosterone. Basal aldosterone levels are indeed increased by Mg deficiency (Nadler et al.,1993).

- Vitamin D3 metabolites. Mg depletion impairs vitamin D metabolism (Rude et.al., 1985). It has been reported that the synthesis of $1,25(OH)_2D_3$ requires Mg *in vitro,* and that Mg deficiency decreases the synthesis of $1,25(OH)_2D_3$ in the kidney (Risco and Traba,1992). Hypomagnesemia decreases the activity of 1-α hydroxylase, which converts $25(OH)D_3$ into $1,25(OH)_2D_3$ (Kanazawa et al., 2007). Serum $1,25(OH)_2D_3$ is low in Mg-deficient humans and rats and reduces the capacity to absorb Ca (Agus ZS, 1999; Rude RK et al., 2003). In addition, an increased catabolism of $1,25(OH)_2D_3$ was influenced by Mg deficiency.

c. Systemic disturbances

Mg deficiency can be also the etiologic factor that exacerbates disease development (Rude, 1998). In human and animal trials, Mg deficiency has been linked to neuromuscular and central nervous manifestations, cardiovascular diseases (atherosclerosis, hypertension, myocardial infarction), diabetes, inflammation and osteoporosis.(Chollet et al., 2001; Konrad and Weber 2003; Laurant and Touyz RM, 2000; Mazur et al., 2007; Schmitz et.al., 2007; Sontia and Touyz, 2007). The earliest manifestations of Mg deficiency are usually neuromuscular and neuropsychiatric disturbances. This includes positive Chovestek's and Trousseau's signs, tremor, fasciculation and tetany, being the latter frequently associated with hypocalcemia.

- Nervous and neuromuscular system. Hypomagnesemia may be responsible for the decreased transmembrane inositol transport, sorbitol accumulation in the intracellular fluids and myo-inositol depletion (Chetan et al., 2003). Moreover, Mg deficiency may increase intracellular Ca

and the concomitant activation of signaling pathways, increasing also NO production which will cause nerve damage (Guerrero-Romero and Rodriguez-Moran, 2006). In the central nervous system, Mg also plays an important role in neuroprotection and antinociception due to its blocking properties in a voltage-dependent manner at the NMDA receptor channel complex.
The mechanism by which Mg affects the neuromuscular system relates to the fact that Mg stabilizes the nerve axon and influences the release of neurotransmitters at the myoneural junction. In Mg deficiency, there is a decreased threshold for axonal stimulation and an increased quantity of neurotransmitter released. Mg is also involved in Ca handling by the muscle cell. With low intracellular Mg, calcium is more readily released from sarcoplasmic reticulum and is reaccumulated more slowly. This results in a muscle that is more prone to contraction in response to a given stimulus and is less able to recover from contraction.

- Immune system. Mg deficiency might be accompanied by the activation of cells such as macrophages, neutrophils and endothelial cells (Mak et al., 1997; Malpuech-Brugere et al., 2000). In fact, macrophages have been found in the peritoneal cavity of Mg-deficient rats. These macrophages seem to be activate endogenously and could contribute, at least in part, to the increased production of proinflammatory cytokines. Polymorphonuclear (PMN) cell number and function has also been shown to be altered in rats fed with a Mg deficient diet together with the characteristic inflammatory response. In fact, an increased PMN number and an increased phagocytosis has been described in Mg-deficient compared to control rats. Mg deficiency also seems to accelerate thymus involution. One of the most remarkable results, regarding effects of Mg deficiency on the organism, is the higher level of apoptosis shown in thymuses from Mg deficient rats as compared with controls (Malpuech-Brugere et al., 2000). Clinical signs of inflammation, splenomegalia and leukocytosis are also accentuated in Mg-deficient rats (Mazur A et al., 2007).

- Cardiac arrhythmias. Mg deficiency can affect cardiac electrical activity, myocardial contractility and vascular tone. Mg deficiency causes some electrocardiographic (ECG) changes. On ECG moderate Mg deficiency may result in flattening of T-wave, shortening of the QT segment and prolonging of the PR and QRS intervals. In severe Mg deficiency, all of the above alterations may occur. This could be explained by the coexisting hypokalemia and other electrolytes disturbance (see above) (Fawcett et al., 1999). Mg depletion renders the heart more susceptible to various arrhytmogenic stimuli probably because of its effect on maintaining intracellular potassium. Mg is necessary for Na/K ATPase, which is responsible for active transport of K intracellulary during phase 4 of the action potential. Mg also is involved in

regulating the potassium influx through other potassium channels. Mg appears to regulate the outward movement of potassium in myocardial cells. Therefore, a deficiency of myocardial Mg can lead to reduced intracellular K due to a less efficient Na/K-ATPase system and the loss of inward rectification (Matsuda H, 1991; White RE et al., 1989). Because the resting membrane potential is determined in part by the intracellular K concentration, decreased intracellular K results in a less negative resting membrane potential. The results is a prolungation of the QT interval and enhanced vulnerability for ventricular arrhythmias.

- Vascular diseases. Some researchers have hypothesized that Mg deficiency contributes to several cardiovascular disturbances through the infiammatory process (Maier, 2003). Indeed, in VSMC, low Mg upregulates inflammatory mediators through the activation of nuclear factor kappa B (NFkB) (Laurant P and Berthelot, 1992, 1994; Altura et al., 2003). Therefore, atherosclerosis may be induced by inflammatory response and changes in lipoprotein metabolism. These events enhance the expression of adhesion molecules, stimulate the proliferation of smooth muscle cell, macrophages, lymphocytes T and increase interaction between modified lipoproteins (Mazur et al., 2007). Experimental Mg deficiency is characterized by increased plasma levels of triglycerides, cholesterol, VLDL, LDL, apolipoprotein B and reduced HDL. Moreover, Mg deficiency increases free radical production which may induce lipid peroxidation and increase platelet aggregation (Swaminathan, 2003). Hypertension after prolonged deficiency may be the consequence of arterial structural changes and oxidative stress (Bukoski, 2002; Touyz RM et al., 2002). Oxidative stress inactive NO, an event which may contribute to hypertension observed in chronic Mg deficiency (Shivakumar and Kumar, 1997). In addition, Mg directly influences vascular tone by regulating endothelial function and vascular smooth cells (VSMC) function (Maier JAM et al., 2004; Laurant P and Berthelot, 1992 -1994).
Interestingly, oral Mg therapy has been associated with significant improvement of endothelial function in patients with coronary artery disease (Shechter et al., 2000) and with a decrease of plasma concentrations of triglycerides, VLDL and apo-B (Rasmussen et al., 1989).

- Metabolic syndrome. The metabolic syndrome is a cluster of common pathologies: obesity, insulin resistance, dyslipidemia and hypertension. Recently, experimental, clinical, and epidemiological data have provided strong evidence that dietary Mg intake is inversely associated with the risk for metabolic syndrome and its components (Guerrero-Romero and Rodrìguez-Moràn, 2006). This is not surprising since Mg deficiency impairs glucose and lipid metabolism, alters immune response, and increases free radical production, all important factors

in the pathogenesis of the metabolic syndrome. The relationship between Mg and metabolic syndrome has been recently extensively reviewed (Belin and He, 2007).

- Osteoporosis. Mg deficiency has been implicated in osteoporosis (Rude RK, 1998). The relationship between osteoporosis and hypomagnesemia is still controversial. Mg deficiency and resulting hypomagnesemia cause osteoporosis and bone fractures through dysfunction of parathyroid hormone (PTH) and vitamin D action (Rude and Gruber, 2004). Infact, acute changes in [Mg]e will influence PTH secretion in a manner qualitatively similar to Ca by binding to the calcium-sensing receptor. Most patients with hypocalcemia due to Mg deficiency have low or inappropriately normal serum PTH levels and the administration of Mg will result in an immediate rise in the serum PTH level.

However, other studies have shown no relationship between Mg deficiency and osteoporosis (Michaelsson et al., 1995). It is known that the bone serves as a reservoir to maintain the [Mg]e concentration. In situation of chronic Mg depletion, Mg content in bone is thought to decrease. Some authors have not seen hypomagnesemia after dietary deprivation of Mg. Presumably loss of bone Mg causes no symptoms and enormous deficits might occur in this organ if the depletion developed slowly (Dunn and Walser, 1996).

Individuals affected by primary hypomagnesemia due to renal Mg wasting inherited in an autosomal dominant manner have been reported to demonstrate significant reductions in bone mineral density (BMD) (Kantorovich et al., 2002). Experimental Mg deficiency results in decrease bone growth, increased bone resorption, decreased bone volume, increase in skeletal fragility and in a delay of apatite crystal formation and growth (Rude RK, 1998; Rude RK et al., 1999).

2. MAGNESIUM DEFICIENCY IN ANIMAL MODELS

Several animal models (hamsters, rats, mice, dogs and primates) have been used to simulate clinical hypomagnesemia and all have exhibited histologic, electrical and/or functional abnormalities of the cardiovascular system trough the activation of an inflammatory process (Rayssiguier Y et al., 2001). Animals placed on Mg-restricted diets display progressive cardiovascular lesion formation, heightened cardiac inflammatory cell infiltration, neuromuscular hyperexcitability, arrhythmias, and increase oxidative stress with decreased levels of endogenous antioxidants and higher plasma levels of prooxidant metals (Kramer JH et al., 1994; Kramer JH et al., 2009). Moreover, preexisting Mg deficiency was shown to amplify myocardial vulnerability to toxic agents and imposed stresses. When the imposed stress was ischemia/reperfusion, dogs on a Mg-restricted diet developed larger infarcts than those on a Mg normal diet. In addition, hearts from Mg deficient rats exhibited lower recovery of cardiac function compared with the nondeficient group.

The first observations of clinical symptoms of inflammation in Mg deficient rats have been published in 1930s (Kruse HD et al., 1932). A characteristic allergy-like crisis with erythema, hyperemia, and edema occurs spontaneously in Mg deficient rats (Classen CU et al., 1993).

Mg deficient rodents also exhibited elevation of circulating inflammatory mediators, which preceded significant leucocyte infiltration of the heart. Circulating levels of proinflammatory neuropeptides, substance P (SP), and calcitonin gene-related peptide were probably emanated from sensory-motor neurons. These neuropeptides may trigger inflammatory/oxidative events which induce cardiomyopathy (Weglicki WB et al., 1994). In severe Mg deficiency rats, elevations of plasma SP preceded the peak of leukocytes and inflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor alpha (TNF-α), histamine, prostaglandin E2, gamma-interferon. MgD also induced reactive nitrogen species production and nitric oxide synthase (NOS) upregulation in rat model (Kramer JH et al., 2009).

Vitale and coworks, in 1950s, started to study the effect of magnesium deficiency in the pathogenesis of atheroscelrosis in rats, dogs and nonhuman primates, and saw a regression of these lesion after administration of high level of dietary magnesium (Vitale J.J et al., 1957). More recently Mg has been found to modulate atherogenesis and atherosclerosis in rabbit (Altura et al, 1990). Magnesium supplementation suppresses atherogenesis in low-density (LDL)-receptor-deficient mice and induces favorable antiatherogenic changes in apoE knock out mice (Ravn HB et al., 2001).

Indeed, several lines of evidence support a role for inflammation as the major origin of oxidative stress. However, in the first approach, the inflammatory response has been proposed to be consecutive to oxidative damage during Mg deficiency. Indeed numerous works from several groups have reported indices of oxidative stress in Mg deficiency animals: enhanced tissue, erythrocyte and lipoprotein peroxidation, oxidative modifications of proteins, reduced antioxidant status and increased plasma nitric oxide (NO).

These observations indicate that Mg deficient animals are more sensitive to oxidative stress than controls. Both macrophages and neutrophils generate superoxide anions in response to various stimuli by the means of a NADPH oxidase. It has been shown that the basal neutrophil activity of Mg deficient rats was higher than that of controls (Bussiere et al., 2002). Neutrophils from Mg deficient rats were more responsive to activation by immune stimuli than those of control animals. Such activation was found to be blocked by the administration of a substance P receptor blocker in the Mg deficient rat (Mak IT et al., 2003). This result further supports the neurogenic inflammation linkage with oxidative stress during Mg deficiency.

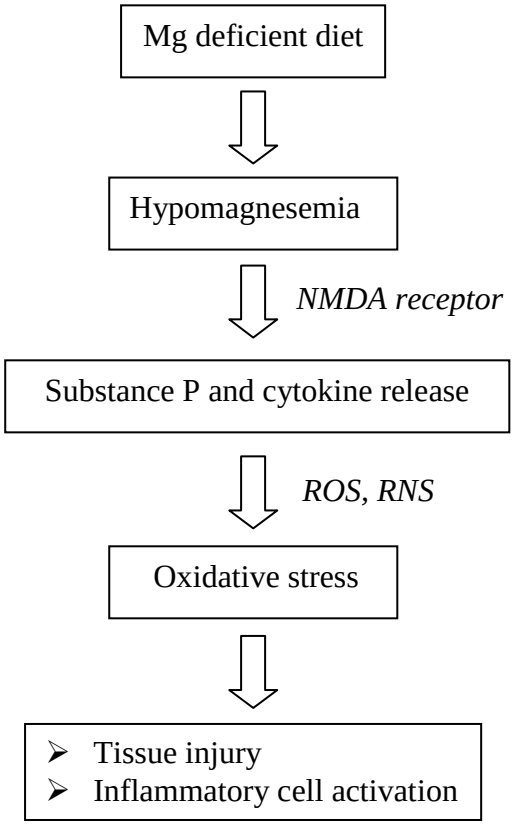

Figure 2 : Summary of the events described in Mg deficient animals.

3. MAGNESIUM, INFLAMMATION AND ENDOTHELIAL CELLS

Endothelial cells actively contribute to inflammation by elaborating cytokines, synthesizing chemical mediators and expressing adhesion molecules which interact with leucocytes thus facilitating their passage into the nearby tissues. In addition, the endothelium itself is sensitive to cytokines which profoundly affect its behavior and play a role in promoting dysfunctions implicated in the pathogenesis of different diseases.

Because of their strategical location at the interface between blood and vessels, endothelial cells are readly exposed to various signals, among which low Mg, which provokes an inflammatory response. Since they are highly heterogenous, it is noteworthy that both micro- and macro-vascular cells are sensitive to fluctuations of extracellular concentrations of Mg.

Microvascular cells are the protagonists in the vascular changes during inflammation, being involved in increasing the caliber and the permeability of the vassels in the beginning, while later in the process they facilitie leukocyte chemotaxis. Through, the induction of endothelial nitric synthase (eNOS), low magnesium promotes the synthesis of nitric oxide (NO) which acts in a paracrine fashion to stimulate smooth muscle cells guanyl cyclase to produce 3',5'-cyclic monophosphate, responsible of relaxation of the blood vessels (Ignarro JJ., 1990). Low extracellular Mg also induces the expression of vascular cell adhesion molecule (VCAM) (Bernardini D et al., 2005), a marker of inflammation which binds the integrin VLA-4 (very late activation antigen-4), expressed by monocytes and most of the lymphocytes. Focal adhesion of leukocytes to the microvascular is a key step in inflammation and immune response. In addition, Mg deficiency upregulates interleukin (IL)-1α and IL-6, pleiotropic cytokines implicated in acute phase responses and inflammation (Bernardini D et al., 2005). Since, IL-6 levels are high in animals fed Mg deficiency diet, it is conceivable that microvascular cells are an important source of this cytokine under these conditions.

Also Macrovascular endothelial cells are actived by low Mg to elicit an inflammatory response (Maier JAM. et al., 2004). Low Mg increases the adhesion of monocytes to cultured endothelial cells via upregulation of VCAM, induces plasminogen activator inhibitor (PAI)1, augments the levels of pro-inflammatory IL-1α, impairs endothelial proliferation and promotes cellular senescence (Maier JAM. et al., 2004; Killilea and Maier JAM., 2008). This is crucial because atherosclerosis is recognized as a chronic inflammatory disease resulting from the interaction between modified lipoproteins, blood derived cells and the normal components of the arterial wall. Low Mg also increases the transport of LDL across the endothelial monolayer as well as the productionof free radicals which might oxidize the lipoprotein. It was also shown that

endothelial cells cultured in low Mg medium are more sensitive to oxidative stress (Zhou et al.,1999). Another crucial and early step in ateherogenesis is the recruitment of leukocytes to the intima, and low Mg markedly and reversibly upregulates VCAM in macrovascular cells. Once adherent, the leukocyte penetrate the artery wall directed by some chemokines. Interestingly, Mg deficiency induces the synthesis of some of these molecules by endothelial cells. In addition, low extracellular Mg increases endothelial secretion of growth factors and cytokines, which perpetuate cell dysfunction and also affects smooth muscle cell functions (Maier JAM. et al., 2004).

C. OXIDATIVE STRESS

The endothelium, an active biologic organ, contributes to the local balance between pro- and anti-inflammatory mediators, hemostatic balance, as well as to vascular permeability and cell proliferation. The normal endothelium has anticoagulant and anti-inflammatory properties, and regulates vascular caliber by production of nitric oxide (NO), prostacyclin and other vasodilators. An impairment of endothelial-mediated vasodilation characterizes endothelial dysfunction. It is the result of impaired NO activity, mainly due to its inactivation by ROS and increased release vasocostrictor factors. In various diseases the endothelium becomes dysfunctional, promotes thrombosis and inflammation and lose its vasodilator influences (Guzik, 2003). Endothelial dysfunction has been shown to precede vascular diseases and atherosclerotic lesion formation (Schachinger V. et al., 2000).

A major mechanism responsible for such endothelial dysfunction is the excessive production of ROS. ROS can impact on the production of NO, stimulate proinfiammatory gene expression and increase procoagulant mechanisms.

1. OXIDATIVE STRESS, INTRODUCTION.

Oxygen free radical or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognised for being either harmful or beneficial to living system (Valko, Rhodes, Moncol, Izakovic and Mazur 2006).

Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular response to noxia, as for example in defence against infectious agents and in the response to a number of cellular signalling systems.

The harmful effect of free radicals causing potential biological damage is termed oxidative stress and/or nitrosative stress. This occurs in biological systems when there is an overproduction of ROS/RNS on one side and deficiency of enzymatic and non-enzymatic antioxidants on the other. In the other words, oxidative stress resulting from metabolic reactions that use oxygen represents a disturbance in the equilibrium of prooxidants/antioxidants. The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by a mechanism called “redox regulation”. The process “redox regulation” protects living organisms from various oxidative stresses and maintains “redox homeostasis” by controlling the redox status *in vivo* (Dröge, 2002).

Oxidative stress has been implicated in a number of human diseases as well as in the ageing process. ROS can deplete the cellular levels of NO, increase the expression of adhesion molecules (P-selectin, VCAM-1), lipid inflammatory mediators such as platelet-activating factor (PAF), leukotriene B4 and cytokines such as IL-8 and monocyte chemotactic protein (MCP-1). Moreover, ROS might act as a second messenger and have significant effects on the vascular cell signal transduction pathways involving mitogen-activated protein kinase (MAPK), protein kinase C (PKC), extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and NFkB. In addition to these mechanisms, reactive species can modulate ion channels, influence Ca and K concentrations and finally activate matrix metalloproteinases (e.g. MMP-13, MMP-9 and MMP-2) (Figure 5).

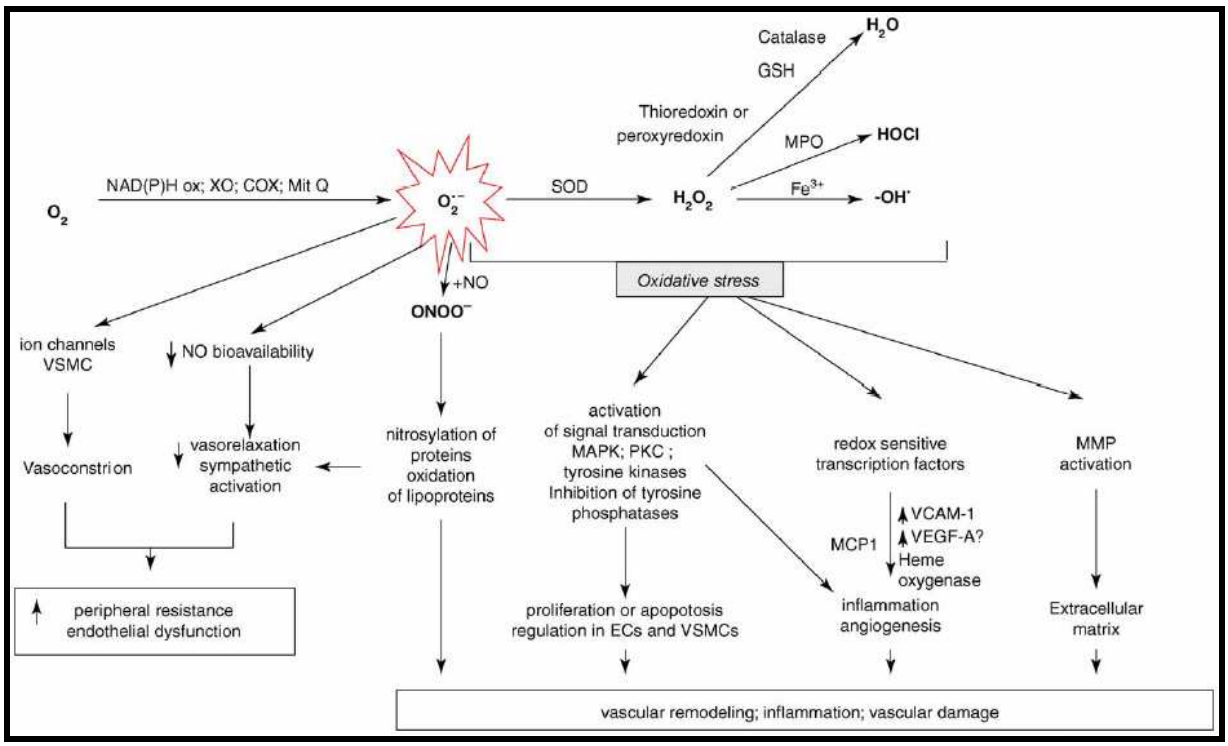

Figure 5: **ROS production, their interactions and biological consequences.**
Superoxide can be produced by numerous oxidoreductases. Superoxide can then rapidly react with NO forming peroxynitrite and leading to loss of NO bioavailability and impaired endothelium-dependent vasodilatation. ROS can stimulate mitogenesis in vascular smooth muscle cells (VSMC), activate other redox-sensitive signaling pathways and trascription factors and also oxidize cellular proteins.

1.1 Reactive oxygen species (ROS)

Reactive oxygen species are produced as intermediates in reduction-oxidation (redox) reactions. ROS are reactive chemical entities comprising two major groups:

- Free redicals (e.g. superoxide [$\bullet O_2^-$], hydroxyl [OH•] ,nitric oxide [NO•])
- Nonradical derivates of O_2 (e.g. H_2O_2, $ONOO^-$, hypochlorous acid HOCl, lipid peroxides LOOH).

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell & Gutteridge,1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radicals.

Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical (Miller et al., 1990). Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme-or metal catalese processes. Nonradical derivates do not contain an unpaired electron and, therefore, are prone to exchanging electrons with other molecules. They are less reactive and more stable with a longer half-life than free radicals.

Of the ROS generated in cardiovascular cells, $\bullet O_2^-$ and H_2O_2 appear to be particularly important.

1.2 Reactive nitrogen species (RNS)

Nitric oxide is a small molecule that contains one unpaired electron and is, therefore, a free radical. NO• is generated in biological tissues by specific nitric oxide synthase (NOSs), which metabolise arginine to citrulline with formation of NO• *via* five electron oxidative reaction (Ghafourifar & Cadenas, 2005). Nitric oxide (NO•) is an abundant reactive radical that plays a key role in the protection exerted by endothelium against coronary disease. NO• not only prevents abnormal constriction of the coronary arteries but also inhibits the aggregation of platelets, the expression of adhesion molecules at the surface of the endothelial cells, and hence the adhesion and penetration of white blood cells, and the release and action of the vasoconstrictor and mitogenic peptide endothelin-1. NO• has a half-life of only a few seconds in acqueous environment, while it has greater stability in an enviroment with a lower oxygen concentration (half-life >15s). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes (Chiueh, 1999). Overproduction of reactive nitrogen species is called "nitrosative stress" (Klatt & Lamas, 2000; Ridnour et al.,

2004). This may occur when generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and inhibit their normal function. During the inflammatory process the cells produce both superoxide anion and nitric oxide that may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$) which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Carr, McCall& Frei, 2000).

$NO\bullet + \bullet O_2^- \longrightarrow ONOO^-$

1.3 Sources of ROS in vascular cells

ROS are produced by all vascular cell types, including endothelial, smooth muscle and adventitial cells and can be formed by numerous enzymes (Figure 6). Enzymatic sources of ROS include the mitochondrial respiration, arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, xanthine oxidase (Cai H et al., 2000), uncoupled nitric oxide synthase (eNOS), NADH/NADPH oxidases (Guzik et al., 2004), and cytochrome P450 (Wolin M.S., 1996), peroxidases, and other hemoproteins.

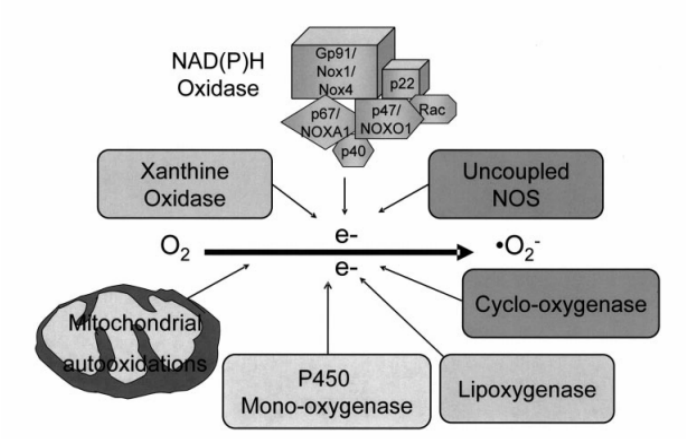

Figure 6: **Enzymatic source of superoxide anion ($\bullet O_2^-$).**

A large body of evidence based on animal models suggests that membrane-specific NAD(P)H oxidase complexes, xanthine oxidase, a dysfunctional eNOS are the major sources of the superoxide anion and •O2⁻ formation in various pre-atherosclerotic conditions (Rajagopalan et al.,1996) (Figure 7). These data have been confirmed in human peripheral arteries as well as in coronary arteries (Spiekermann et al., 2003; Sorescu et al., 2002).

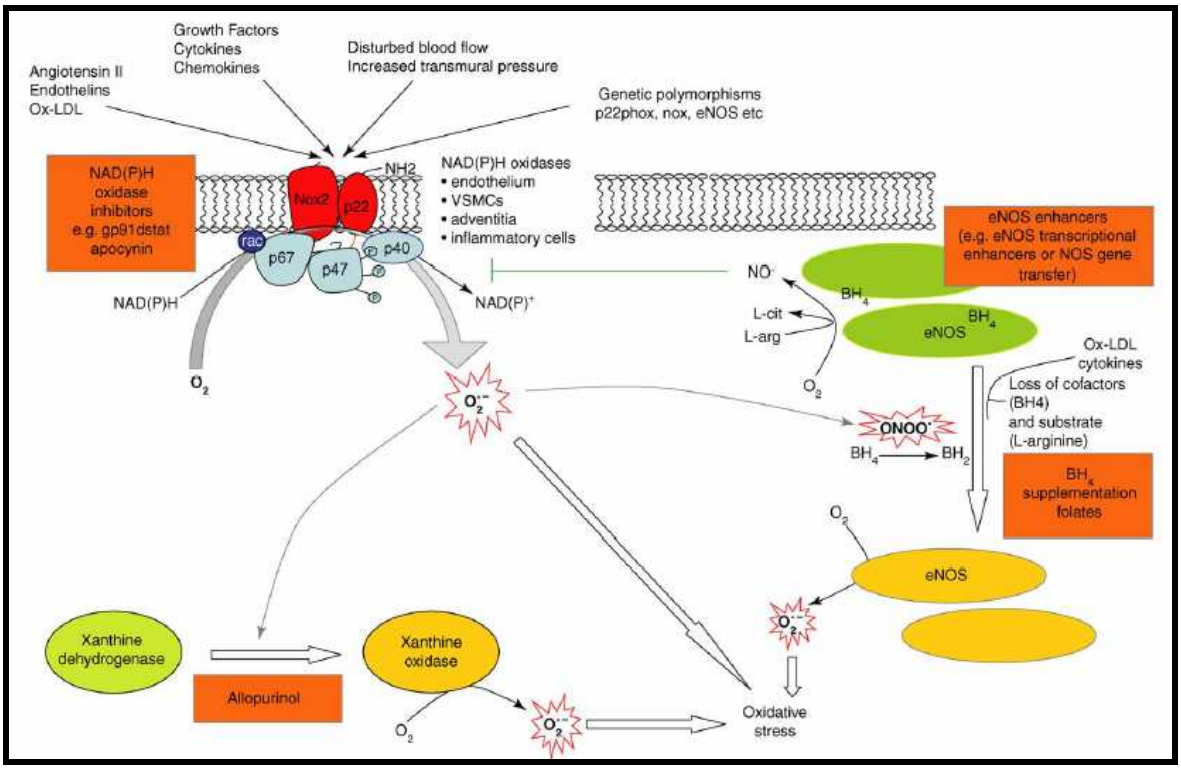

Figure 7: **Role of NADPH, xanthine oxidase and eNOS in the pathogenesis of vascular oxidative stress.**

2. NADPH OXIDASE

NAD(P)H oxidase is multi-subunit enzyme that catalyzes •O2⁻ production by 1-electron reduction of O_2 using NADPH or NADH as the electron donor.

The prototypical NAD(P)H oxidase is that found in neutrophils and has five subunits: p47phox ("phox" stands for *ph*agocyte *ox*idase), p67phox, p40phox, p22phox and the catalytic subunit gp91phox (also termed "Nox2" (Barbior BM., 2004; Chabrashvili T. et al., 2002). In unstimulated cells, p47phox , p67phox and p40phox exist in the cytosol, whereas p22phox and gp91phox are in the membrane, where they occur as a heterodimeric flavoprotein, cytocrome b558. Upon stimulation, p47phox becomes phosphorylated and the cytosolic subunits form a complex that translocates to the membrane, where it associates with cyto-chrome b558 to assemble the active oxidase, which transfers electrons from the substrate to O_2 forming $\bullet O_2^-$ (Touyz RM et al., 2003).

Although NAD(P)H oxidases were originally considered as enzymes expressed only in phagocytic cells involved in host defense and innate immunity, recent evidence based on the discovery of gp91phox homologs indicates that there is an entire family of NAD(P)H oxidase (Geiszt M et al., 2006 and Cave et al., 2006). The new homologs, along with gp91phox are now

designated the Nox family of NAD(P)H oxidase. The family comprises seven members, including Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Griendling KK, 2006). They are expressed in many tissues and mediate diverse biological functions. Nox1 is found in colon and vascular cells and plays a role in host defense and cell growth. Nox2 is the catalytic subunit of the respiratory burst oxidase in phagocytes, but it is also expressed in vascular, cardiac, renal and neural cells. Nox3 is found in fetal tissue and the adult inner ear and is involved in vestibular functions. Nox4, originally termed "Renox" (renal oxidase) because of its abundance in the kidney, is also found in vascular cells and osteoclasts; and Nox5 is a Ca+-dependent homolog, found in testis and lymphoid tissue, but also in vascular cells. Duox1 and-2 are thyroid Noxes involved in thyroid hormone biosynthesis.

In vascular cells the activity and expression of NADPH oxidase can be regulated by cytokines (TNFα, transforming growth factor β, PDGF), hormones, and agonists like ANG II and thrombin (Griendling et al., 2000). Hemodynamic forces, such as oscillatory shear-stress and stretch, can also activate NADPH oxidase activity and expression.

2.1 Role of NAD(P)H oxidase-derived ROS in vascular biology

ROS influence vascular cell growth, migration, proliferation and activation (Touyz RM et al. 2003; Cai H., 2005). Physiologically, NADPH oxidase-derived ROS have been implicated in the regulation of vascular tone by modulating vasodilation directly or indirectly by decreasing NO bioavailability through quenching by $\bullet O_2^-$ to form $ONOO^-$ (Kajiya M et al., 2007; Shimokawa H et al., 2004). ROS, through th regulation of hypoxia-inducible factor1 (HIF-1), are also important in O_2 sensing. In pathological conditions, ROS are involved in inflammation, endothelial dysfunction, cell proliferation, migration and activation, extracellular matrix deposition, fibrosis, angiogenesis, and cardiovascular remodeling, important processes contributing to cardiovascular and renal remodeling in hypertension, atherosclerosis, diabetes, cardiac failure, and myocardial ischemia reperfusion injury. These effects are mediated through redox-sensitive regulation of multiple signaling molecules and second messengers including mitogen-activated protein kinases, protein tyrosine phosphatases (PTP), tyrosine kinases (PTK), proinflammatory factors, ion channels and Ca^{2+} and matrix metalloproteinase (MMPs) (Figure 8).

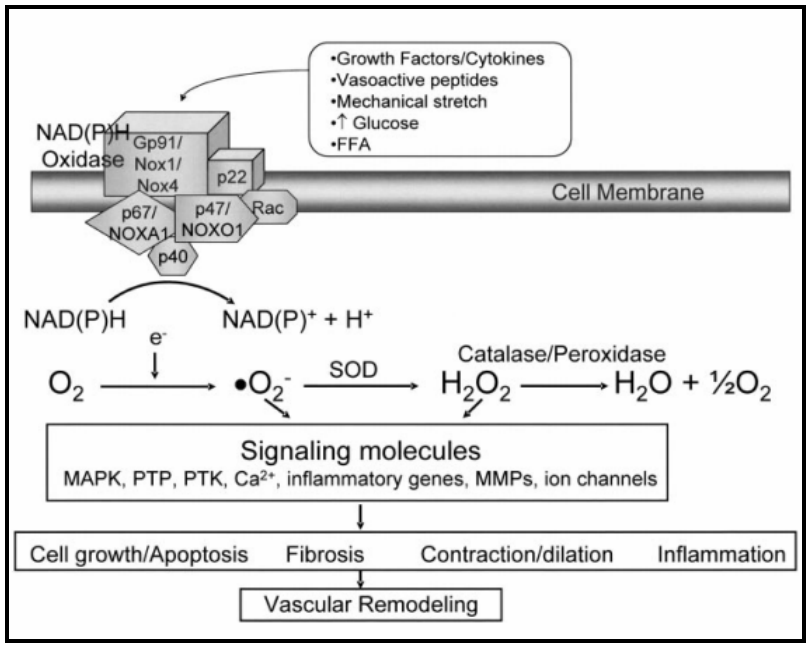

Figure 8: **ROS generation resulting by activation of vascular NAD(P)H oxidase**. ROS influence signaling molecules involved in vascular growth, fibrosis, contraction/dilation and inflammation. These redox-sensitive processes contribute to vascular damage and remodelling in cardiovascular disease. FFA: free fatty acid

3. XANTHINE OXIDASE

Xanthine oxidoreductase (XOR) is part of a group of enzymes known as the molybdenum iron-sulfur flavin hydroxylases. It was first discovered in milk by Schardinger in 1902 and is thought to be involved in reactions that produce ROS such as nitrite which enable newborn infants to overcome gut-associated bacterial gastroenteritis (Hancock JT et al., 2002; Stevens CR et al., 2000). XOR is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, brain and plasma. In the myocardium, it is localized to the capillary endothelial cells (Panus PC et al., 1992). It exists in two inter-convertible forms known as xanthine oxidase (XO) and xanthine dehydrogenase (XDH) and both enzymes consist of two identical subunits. Mammalian XOR is present *in vivo* as the dehydrogenase form but is easily converted to XO by oxidation of the sulfhydryl residues or by proteolysis. Both XO and XDH can oxidize NADH which results in ROS formation. The source of XO is not completely clear is present in the vascular endothelium and can be an additional source of vascular superoxide. Stimuli such as hypoxia and reoxygenation, cytokines, and oscillatory shear-stress upregulate endothelial XO transcription and activity (Berry CE et al., 2004). XO is a important source of ROS in a variety of pathophysiological states, including hypertension, atherosclerosis, ischemia-reperfusion and heart failure and coronary artery disease (Spiekermann S et al., 2003).

4. ENDOTHELIAL NO SYNTHASE

NOS can also contribute to ROS production, since all three NOS isoforms (neural NOS or nNOS, inducibile NOS or iNOS and endothelial NOS or eNOS) have been shown to be susceptible to the "uncoupling" that leads to the formation of $\bullet O_2^-$ and H_2O_2. In the absence of its cofactor tetrahydrobiopterin (BH_4) or its substrate (L-arginine), the endothelial NO synthase (eNOS) generates $\bullet O_2^-$ instead of NO (Alp N.J and Channon 2004). BH_4 plays an essential role in transfer of electrons from the prosthetic heme group of NOS to L-arginine, ultimately leading to NO and citrulline production. In the absence of BH_4, the $Fe^{II}OO$ intermediate (formed during NOS catalysis) yields $\bullet O_2^-$. Uncoupling of eNOS in the endothelium may lead to oxidative stress and endothelial dysfunction via at least three mechanisms. First, the enzymatic production of NO is diminished, allowing the radicals that it normally might react with to attack other cellular target. Second, the enzyme beings to produce $\bullet O_2^-$, contributing to oxidative stress. Finally, it is likely that eNOS can become partially uncoupled, such that both $\bullet O_2^-$ and •NO are produced simultaneously. Under this circumstance, eNOS may become a peroxynitrite generator, leading to a dramatic increase in oxidative stress (Cai and Harrison, 2000).

D. MAGNESIUM TRANSPORT

Intracellular Mg is tightly regulated by precise control mechasnisms that involve Mg influx and efflux across the cell membrane. Small changes of [Mg]i lead to significant effects on signaling pathways that regulate cellular functions. Although Mg is such an abundant cytosolic cation and is so important in biological processes, little was known about the transport mechanisms regulating Mg homeostasis until recently. Transporters and exchangers that have been implicated in transmembrane Mg transport include the Na/Mg exchanger, the Mg/Ca exchanger, and the recently identified transient receptor potential melastatin 6 and 7 channels (TRPM6 and TRPM7) (Monteilh-Zoller MK et al., 2003; Romani AM, 2007; Schlingmann KP et al., 2007; Schmitz C et al., 2005).

- **Mg efflux**

Mg efflux involves Na-dependent and Na-independent systems. Na-dependent Mg transport occurs via the Na/Mg exchanger, whereas in the Na-independent mechanisms Mg is exchanged with extracellular ions, including Ca, Mn, and Cl (Ca/Mg exchanger, Mn/Mg antiporter, Cl/Mg cotransporter). When [Mg]i is increased, e.g. by Mg loading, Mg efflux is induced via Na/Mg antiport as long as [Mg]i is normalized. On the other hand, when [Mg]i is reduced, Mg is taken up until normal [Mg]i is reached (Romani AM, 2007). Although some uncertainties remain concerning the properties of Na/Mg exchange, the transport is stimulated by intracellular ATP, inhibited by amiloride and modulated by cytoplasmic Na and Ca. The Na/Mg exchanger has been demonstrated in many cardiovascular cell types, including VSMCs and cardiomyocytes. In these cells, it is regulated by multiple factors important in vascular biology and in hypertension such as ANGII, vasopressin, isoproterenol, ET-1, and insulin. Cardiac Na/Mg antiport stochiometry appears to be 1Na: 1Mg.

Na-independent Mg extrusion pathways have been demonstrated only in erythrocytes and hepatic cells, but not in vascular cells (Gunther T et al., 1992; Gunther T, 2007).

- **Mg influx**

Unlike other major cellular ions like Na, Ca and K, little is known about system that regulate Mg influx. Functional studies have suggested that Mg enters cell through Mg/anion cotransport utilizing the electrochemical gradient of Na and through cation channels. At least seven transmembrane Mg channels have been cloned. The mitochondrial RNA splicing 2 protein

(Mrs2p) was the first Mg transporter characterized (Kolisek M. et al., 2003; Bui DM et al., 1999). Numerous molecules with Mg transport capabilities have been identified through a screen designed to discover genes upregulated under hypomagnesemic conditions including the human solute carrier family 41, members 1 and 2 (SLC41A1, SLC41A2) channels, Mg transporter 1 (MagT1), and Ancient Conserved Domain Protein 2 (ACDP2). The most surprising players in the regulation Mg homeostasis are the two transient receptor potential channel family members TRPM6 and TRPM7, which were recently characterized (Table 3).

However, biological significance and mechanisms of regulation of all these transporters have yet to be described.

Investigation of molecular causes underlying hereditary disease associated with hypomagnesemia led to the cloning and characterization of a paracellin-1 (claudin 16) a member of the claudin family and located in the kidney (Simon DB et al., 1999). Paracellin-1 mutations are associated with hereditary disease called hypomagnesemia, with hypercalciuria and nephrocalcinosis characterized by massive renal Mg and calcium wasting leading to end-stage renal disease.

Transporter	Cells/tissue
TRPM6	Kidney tubules Vascular smooth muscle cells
TRPM7	Vascular smooth muscle cells Cardiomyocytes Kidney tubules
SLC41A1	Heart Kidney
SLC41A2	Kidney
MagT1	Distal convoluted tubule cells
ACDP2	Kidney cortex
Paracellin-1	Ascending limb, loop of Henle
Mrs2p	Inner mitochondrial membrane

Table 3: Mg transporters in cardiovascular and renal cells involved in Mg efflux and influx.

1. THE TRANSIENT RECEPTOR (TRP) CHANNELS

TRP channels is a large, diverse superfamily of proteins that are expressed in a great variety of multicellular organisms, including worms, fruit flies, zebrafish, mice and humans. They have been detected in most tissues and cell types including electrically excitable and non-excitable cells (Fleig and Penner, 2004; Venkatachalam and Montell, 2007). Drosophila TRP is the first and founding member of the TRP superfamily of cation channels. The Drosophila carrying the TRP mutation presented a transient instead of a sustained response to light (Montell C, 2005). The flies presented impaired vision because of the lack of a specific Ca influx pathway in the photoreceptors (Venkatachalam and Montell, 2007).

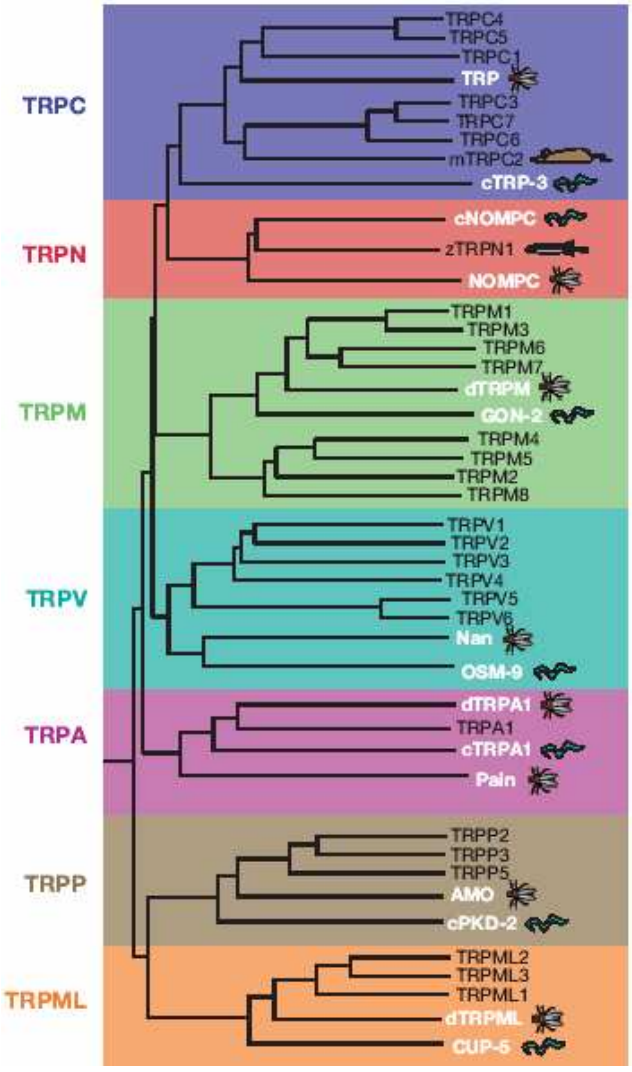

Figure 9: Phylogenic tree showing the relatedness of the TRP proteins.
The dendogram of vertebrate TRPs includes mostly human TRPs, except for mouse TRPC2 and zebrafish TRPN1. White text and cartoons highlight the TRP proteins from worms and flies. One C.elegans and one Drosophila member of each subfamily are included. From Venkatachalam and Montell, 2007.

TRP have a similar topology to voltage-gated ion channels, possess at least six-transemembrane domains and may assemble homo-or heterotetramers to form cation selective channels (Pedersen et al., 2005). The NH_2 and COOH termini of TRP proteins are thought to be located intracellularly and a putative pore-forming region is contained between the transmembrane domains 5 and 6 (Venkatachalam and Montell, 2007). It has been demonstrated that all TRP channels are cation channels but the permeability for different mono- or divalent cations has shown to vary between them (Clapham DE, 2002) (Figure 10).

They are activated by multiple stimuli, including physical (temperature, voltage, mechanical factors), chemicals factors (pH, ions), humoral factors (bradykinin, ANG II, aldosterone), and signaling molecules (phospholipase C, protein kinase C). Based on sequence identity, the TRP protein superfamily is represented by seven protein subfamilies (see Figure 9): N (no mechanoreceptor potential C or NOMPC), A (Ankyrin and TRPA1), P (Polycystin, TRPP1-TRPP3), ML (Mucolipin, TRPML1-TRPML3), C (Canonical TRPC1-TRPC7), V (Vanillioid, TRPV1-TRPV6) and M (Melastatin TRPM1-TRPM8).

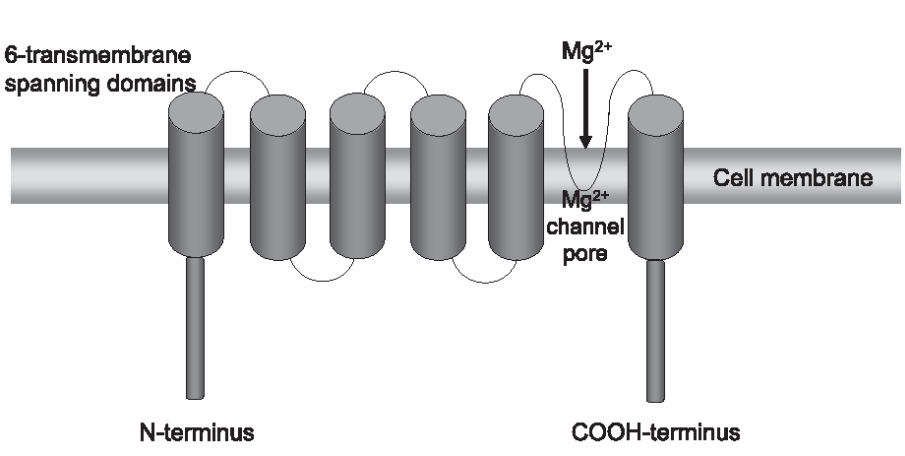

Figure 10: **Proposed membrane topology and putative sites important for Mg transport**.

1.1 TRPC and TRPV channels

The TRPC (TRPC1-TRPC7) channels are non selctive Ca-permeable cation channels and are ubiquitous. VSMCs express mainly TRPC1, TRPC4 and TRPC6, which have been implicated in regulating vascular tone. The TRPV family compries four groups: TRPV1/TRPV2, TRPV3, TRPV4, and TRPV5/6 and is involved mainly in nociception. In the vasculature, activation of TRPV1 and TRPV4 channels induces vasodilation, and TRPV2 may behave as a mechanotransducer (Ionue R et al., 2006).

1.2 TRPM channels

The melastatin-related TRP subfamily was named on the first discovered member, melastatin1 (TRPM1), the gene of which was identified from melanomas (Duncan LM et al., 1998). The mammalian TRPM subfamily consists of eight members which share ~20% amino acid identities to TRPC channels. TRPM proteins comprise six transmembrane segments and cytoplasmic N- and C-terminal domains. Similarly to TRPCs, TRPM proteins have a TRP domain in their C-terminal. The total amino acid lenghts (1000-2000 aa) and sequences of the C-terminal regions of these proteins vary considerably. Based on similarities of aminoacid sequences, members of TRPM family are divided into four gropus: TRPM1/3, TRPM2/8, TRPM4/5 and TRPM6/7 (Fleig and Penner, 2004; Pedersen et al., 2005; Venkatachalam and Montell, 2007).
TRPM channels exhibit highly varying cation permeability, from Ca impermeable (TRPM4/5) to highly Ca and Mg permeable (TRPM6/7).

- TRPM2 is a chanzyme with a C-terminal ADP-ribose pyrophosphatase domain. TRPM2 is expressed mainly in the brain and is a non selective cation channel permeable mainly to Ca, Na, K and Cs. It forms a Ca permeable non specific cation channel that is activated by intracellular ADP-ribose, pyrimidine nucleotides and NAD. This channel forms a cellular redox sensor and is activated by peroxide or other agents that produce reactive oxygen and nitrogen species (Venkatachalam K and Montell C, 2007).
- TRPM3 is found in kidney and brain and forms a constitutively active Ca and Mn permeable channel when expressed exogenously in cultured cells (Grimm C et al., 2003). The spontaneous TRPM3 currents are enhanced by hypotonic extracellular solutions and cell swelling. Alternatively, TRPM3 might be activated by store depletion or sphingolipidis (Venkatachalam K and Montell C, 2007).
- TRPM4 exhibits the highest expression in heart, pancreas, and placenta and TRPM5 is found in tongue, lungs, testis, brain and gastrointestinal tract. TRPM4 and TRPM5 are unusual among

the TRP superfamily in that they are voltage-modulated, Ca activated and permeable to monovalent cation but impermeable to Ca. The monovalent selectivity is dictated by a short acidic strech of six aminoacids in its pore loop (Venkatachalam K and Montell C, 2007).

- TRPM8 is expressed in sensory nerves and the prostate and acts as a plasmalemmal Ca channel and as an intracellular Ca release channel. It is activated by moderately cold temperature, by pharmacological agents evoking a “cool” sensation such as menthol, eucalyptol, and icilin (McKemy et al., 2002).
- TRPM6 and TRPM7 channels have recently been identified as essential for Mg and Ca homeostasis. These proteins have unusual architectures containing a Mg-permeable pore fused to a kinase domain at the COOH terminus, hence termed “chanzymes” (channels plus enzyme) (Schlingmann KP et al., 2002).

TRPM6 has been shown to play in important role in Mg homeostasis as it is localized in the intestinal ephitelia and in the distal convoluted tubule, where final excretion of Mg is determined. TRPM7 is ubiquitously expressed and represents a primarily Mg permeable ion channel required for the cellular uptake of Mg. Both channels are shown to be functionally not redundant, since mutations of one gene do not compensate the deficiency of the other (Monteilh-Zoller MK et al., 2003).

2. TRPM7

TRPM7 is a divalent specific cation channel which is permeable to virtually all physiological divalent cation including Mg and trace metals such as Ni (Monteilh-Zoller MK et al., 2003). It is a protein that contains 1863 aminoacids with a calculated molecular weight of roughly 212.4 KDa and with a protein kinase domain (aa1580-1863) whose functional role remains controversial (Figure 11). TRPM7 is ubiquitously expressed and represents a primarily Mg-permeable ion channel required for the cellular uptake of Mg. Higher expression in kidney and heart has been observed (Montell, 2005). Is also expressed in lymphoid-derived cell lines, hematopoietic cells, granulocytes, leukemia cells, and microglia, vascular smooth muscle cells (He et al., 2005; Schmitz et al., 2003).

TRPM7 appears to be responsible for general cellular Mg influx in virtually every cell type. It may serve potentially both as Mg uptake mechanism and as a Mg sensor. Channel knockdown has been shown to lead to cell death, to inhibit cell proliferation, and, depending on the model studied, to inhibit or to enhance cell adeshion (Abed E, Moreau R, 2007; McNeill et al., 2007).

TRPM7 also plays an important role in the regulation of the cell cycle, cytotoxicity in central nervous system ischemic injury, neuronal cell death, defecation rhythm, pacemaking activity (Adachi M et al., 1993).

It has been demonstrated that human mast cells express functional TRPM7 channels that are critical for cell survival. TRPM7 activity is regulated by pH, ATP, lipids and translocation (Venkatachalam K and Montell C, 2007).

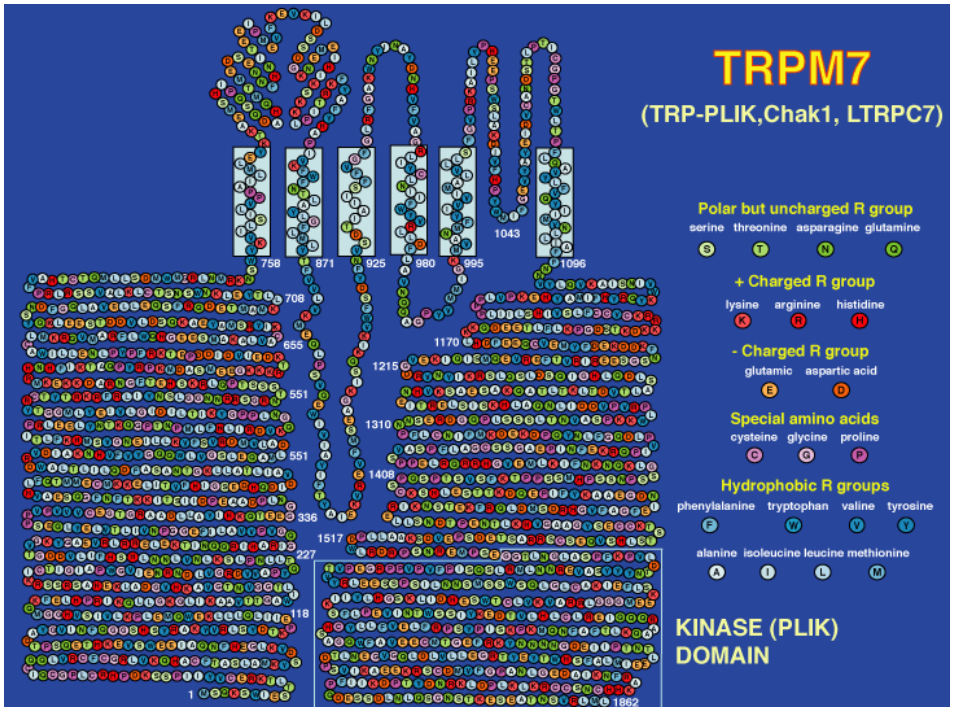

Figure 11. TRPM7 structure

2.1 TRPM7 regulation

TRPM7 regulation by lipids. Contradictory results haven been observed concerning TRPM7 inhibition or not by phospholipids. Clapham and collegues discussed that TRPM7's channel activity requires phosphatidylinositol biphosphate (PIP_2) and its depletion via PLC renders it inactive (Runnels et al., 2002). This was not supported by others (Jiang et al., 2003; Takezawa et al., 2004). They suggest that TRPM7 is modulated by cAMP and PKA, by a mechanism requiring an active TRPM7 kinase. Recently, a different group has shown that the inhibition of TRPM7 currents in response to PLC activation only takes place under low [Mg]i, but that at physiological ionic concentrations, TRPM7 currents are actually activated following PLC activation (Langeslag et al., 2007)

TRPM7 regulation by protons. TRPM7 is upregulated tenfold following a decrease in extracellular pH to 4.0 from physiological pH of 7.4 (Jiang J et.al., 2005). Thus a portion of the TRPM7 pool may be situated in an acidic environment, such as acidic vesicles. H_2O_2 has shown to increase TRPM7 inward currents, but the mechanism is not known. Also it has been observed that protons potentiate TRPM7 inward currents (Jiang J et.al., 2005). At low pH inward current of monovalent cation is increased and this is exacerbated when Ca and Mg concentration is decreased. They explained this feature by a competition for the binding sites in the external pore of the TRPM7 channels, thereby removing the divalent cation block. Mutations in the binding site of Mg and Ca at the pore region eliminate Ca and Mg permeation and abolishes the proton-induced increase currents (Li et al., 2007).

TRPM7 regulation by Mg. TRPM7, like TRPM6, is regulated by changes in cytosolic Mg or Mg-ATP, which explains why TRPM7 and TRPM6 were previously called magnesium-inhibiting channels or MagNuM (magnesium-nucleotide-regulated metal ion channel) (Demeuse et al., 2006). The inhibition of TRPM7 channels by Mg/MgATP is voltage independent. The inhibitory binding site for Mg is unknown; it is probably not whitin the pore but, rather, at the level of kinase domain, since TRPM7 kinase activity requires Mg. Mg nucleotides, such as Mg-ATP and Mg-GTP, have been shown to inhibit TRPM7 channels, but the inhibition has been shown to be attributable to free intracellular Mg.

2.2 TRPM7 and kinase domain

TRPM7 α-kinase exhibit no sequence homology to conventional protein kinase (Ryazanov et al., 1997). The crystal structure of TRPM7 kinase has shown a similarity to the catalytic core of eukaryotic protein kinases as well as to metabolic enzymes with ATP-grasp domains. The kinase domain of the TRPM7 channel apparently phosphorylates itself and other substrates such as annexin1, myosin IIA heavy chain and calpain (Figure 12) (Touyz RM, 2008). TRPM7 kinase contains two serine residues that undergo self-phosphorylation, Ser^{1551} and Ser^{1567}. Annexin-1 belongs to a family of Ca and phospholipid-binding proteins, and has been described as an important endogenous modulator of inflammation (Yang et.al., 2006). It is phosphorylated by TRPM7 on Ser^{5}, but the physiological implication of this phosphorylation remains unclear (Dorovkov and Ryazanov, 2004). It contributes to various cellular processes such as cell migration, growth and differentation, apoptosis, vescicle fusion, lipid metabolism, and cytokine expression (Rescher et al., 2004). Myosin II heavy chain is involved in cell migration, cell growth, apoptosis and cytoskeletal organization. Calpain protease is implicated in the control of

cell adhesion (Touyz RM, 2008). The biological significance of TRPM7-regulated annexin-1, myosin II heavy chain, and calpain remains unclear but through its effects on this molecules TRPM7 might be involved in regulating vascular cell adhesion, migration, and contraction (Clark K. et al., 2006; Su LT et al., 2006).

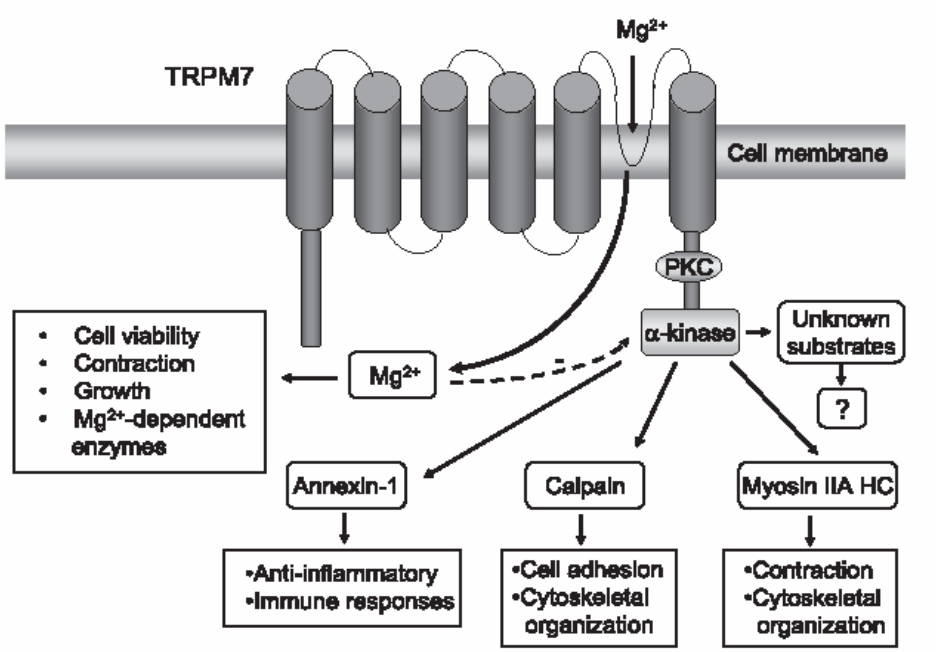

Figure 12: **TRPM7 α-kinase substrates and proposed cellular functions of TRPM7.**
Activation of TRPM7 results in increased transmembrane Mg transport through the channel domain and activation of downstream targets through the α-kinase domain. From Touyz RM, 2008.

The role of the kinase domain of TRPM7 channels has been controversial. Some authors have pointed to the essential role of the kinase domain for channel activation (Runnels et.al., 2001), because point mutations in the kinase domain resulted in non functional channels. Schmitz and coworkers observed that abolishing phosphotransferase activity did not impede channel activation but phosphotransferase-deficient forms of TRPM7 may affect its channel activity by rendering it less sensitive to inhibition by Mg or and Mg-nucleotide, and to regulation by intracellular cAMP (Takezawa et al., 2004). On these bases, they concluded that the kinase domain is necessary for cytosolic Mg/Mg-ATP levels (Schmitz, 2003). Mg-dependent channel suppression is modulated through a functional coupling between the channel gating mechanism and the kinase domain.

2. OBJECTIVES

Mg is referred to as the intracellular divalent cation *par excellence.* Its biological role is extremely versatile as it can serve structural functions as well as dynamic functions. Accordingly, Mg deficiency has been reported to be involved in the pathogenesis of cardiovascular diseases and, in particular, in atherogenesis.

Mg in macrovascular endothelial cells. In cultured macrovascular endothelial cells, low Mg increases tha adhesion of monocytes to the monolayer via upregulation of vascular cell adhesion molecule (VCAM), induces plasminogen activator inhibitor (PAI)1, augments the levels of the pro-inflammatory cytokine interleukin (IL)-1α, impairs endothelial proliferation and promotes cellular senescence (Killilea DW and Ames BN, 2008). Recently, endothelial function has been shown significantly impaired in a model of inherited hypomagnesemia in mice (MgL mice) (Mazur A et al., 2007). All these results point to the fact that low Mg promotes the acquisition of an inflammatory phenotype in endothelium. Since NFkB transcription factors control the inflammatory response and are activated by free radicals, I investigated whether culture in low Mg promotes oxidative stress and activates NFkB in human umbilical vein endothelial cells (HUVEC). Because NFkB activation correlates with marked alterations of the cytokine network, I also studied the secretion profile of inflammatory molecules in cells grown in low Mg. My results highlight molecular events that contribute to the pro-atherogenic effect of Mg deficiency.

Mg in microvascular endothelial cells. MEC contribute to inflammation by elaborating cytokines, synthesizing chemical mediators and expressing adhesion molecules which bind leukocytes, thus facilitating their passage in the nearby tissues. In addition, also in the microvasculature the endothelium itself is sensitive to cytokines which profoundly affect its behavior. Low extracellular Mg affects also microvascular endothelial cells. In murine microvascular cells, it has been reported that low Mg induces the synthesis of vascular cell adhesion molecule (VCAM), a marker of inflammation which binds the integrin VLA-4 (very late activation antigen-4) expressed by monocytes and most of the lymphocytes. Focal adhesion of leukocytes to the microvasculature is a key step in inflammation and immune response. In addition, Mg deficiency upregulates IL-1α and IL-6, pleiotropic cytokines implicated in acute phase response and inflammation (Bernardini D. et al, 2005).

Because MEC are important players in inflammation and angiogenesis, I asked whether different concentrations of Mg could affect the behavior of human MEC *in vitro*. My results demonstrate that culture in low Mg affects human MEC with some differences in respect to HUVEC.

TRPM7, a Mg transport channel. The novel Mg transporter TRPM7, a ubiquitously expressed protein with the feature of being both a functional ion channel and kinase, is a critical regulator of Mg homeostasis in vascular cells. Because endothelial cells are very sensitive to extracellular concentrations of Mg, we have investigated the expression and the role of TRPM7 in human macro- and microvascular endothelial cells. I show that the regulation of TRPM7 expression is very different in human MEC vs HUVEC. By siRNA I also demonstrate completely different effects of TRPM7 silencing in HUVEC vs MEC.

3. MATERIALS AND METHODS

3.1 CELL CULTURE

HUVEC

Human umbilical vein endothelial cells (HUVEC) were from the American Type Culture Collection and cultured in M199 containing 10% fetal bovine serum, 1mM glutamine, 1mM penicillin and streptomycin, Endothelial Cell Growth Factor (ECGF 150 µg/ml), 1mM sodium pyruvate and heparin (5 units/ml) on 2% gelatin-coated dishes. A Mg free medium was purchased by Invitrogen (Milano, Italy) and utilized to vary the concentrations of Mg by the addition of $MgSO_4$ and in some experiments by the addition of $MgCl_2$ or MgPidolate (Mg salt of the L-pyrrolidone carboxylic acid). On the basis of previous reports (Maier JAM et al., 2004; Malpuech-Brugere, 2000; Malpuech-Brugere, 1999), HUVEC were cultured in medium containing 0.1 or 1.0 mM Mg, being the latter the physiological concentration. In some experiments also 5.0 mM Mg containig medium was used. In all the experiments the cells were seeded in growth medium; after 24 h, the medium was changed to expose the cells to either 0.1 or 1.0 mM Mg. In some experiments HUVEC were exposed to lipopolysaccaride (LPS) (1µg/ml) and trolox (40µM), (all from Sigma Aldrich, St Louis, MO).

MEC

Human dermal microvascular endothelial cells (MEC) were from the American Type Culture Collection (Atlanta USA) and routinely cultured in MCDB131 containing 10% fetal bovine serum, 1mM glutamine, 1mM penicillin and streptomycin, 0.1mM hydrocortisone and EGF 10 ng/ml on 2% gelatin-coated dishes. To study the effects of Mg, MEC were cultured in a Mg free medium containing 0.1 or 1.0 mM Mg by addition of $MgSO_4$. In all the experiments the cells were seeded in growth medium and after 24h the medium was changed to expose the cells to either 0.1mM or 1.0mM Mg and, in some experiments, also 5.0mM Mg.

- PROLIFERATION ASSAY

For proliferation assays, the cells were seeded a low density (7500/cm^2) and cultured in growth medium 0.1 or 1.0 mM Mg with or without antioxidants (trolox 40µM, resveratrol l0µM and apocynin 10µM). At different time intervals (every 24h), the cells were trypsinized, stained with a trypan blue solution (0.4%) and the viable cells were counted using a Burker chamber.

- MIGRATION ASSAY

Confluent HD-MEC were cultured in the presence of different concentrations of Mg for 2 days. Migration was determined using an *in vitro* model of wound repair as previously described (Baldoli E et al., 2010). After wound, the monolayer was washed and incubated for 24 additional hours in the corresponding concentration of Mg, before staining with crystal violet.
HUVEC and MEC were grown and transfected to silence TRPM7 (see after) in medium containing 1.0mM Mg. After 24h from transfection the monolayer was wounded, stimulated with FBS and incubated for 16 additional hours.
The wound area was calculated by the ImageJ software and expressed using an arbitrary value scale. The experiments were performed in triplicate. Data are shown as the mean +/- standard deviation (SD).

3.2 GENE SILENCING

To silence TRPM7 expression, we utilized commercial siRNAs (0.2 $\mu g/cm^2$) designed by Qiagen which are 19-22 nucleotide long, double-stranded RNA molecules with two to three nucleotide overhangs. The siRNAs were transfected in sub-confluente HUVEC and MEC using HiPerfectReagent (Quiagen) according to the manufacturer's instructions. 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 in RIPA buffer.
In some experiments the cells transfected with TRPM7 siRNA were trypsinized, stained with a trypan blue solution (0.4%) and the viable cells were counted using a Burker chamber.
Transfection without non silencing siRNAs was used as control.

3.3 CELL CYCLE ANALYSIS

Cell pellets were treated with 0.2 mg/ml propidium iodide in Hank's balanced salt solution containing 0.6% NP-40 and RNAse (1mg/ml) and incubated in the dark at room temperature for 30 minutes. Upon filtration, cell suspensions (10^6 /ml) were analyzed for DNA content on a Coulter EPICS 753 flow cytometer (Beckman Coulter, Miami, FL). The percentage of cells in each phase of the cell cycle was determined by using a Multicycle software version 2.53 (Phoenix Software, San Diego, CA).

3.4 MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

Intracellular reactive oxygen species (ROS) were estimated by using an oxidationsensitive fluorescente probe, 2'7'-dichlorofluorescin diacetate (DCFH-DA Sigma-Aldrich) methods, based on the ROS-dependent oxidation of DCFH-DA to DCF (2'7'-dichlorofluorescin). The diacetate form of DCFH is taken up by the cells, where it is metabolized by intracellular esterases so that it remains "trapped" intracellulary and, in the presence of ROS, it is oxidized to DCF.

HUVECs plated in 96-well plates were grown to confluence in complete M199 media and exposed for 30 min or 1 h to different Mg concentration in complete growth media. Before adding the probe, the medium was removed and the cells were washed by PBS. Then, 200µl DCFH-DA (25µM) were added for 30 min at 37°C in the dark. The cells were washed with PBS and in some experiments were stimulated with H_20_2 (200 µM). Intracellular ROS production was measured with the Perkin Elmer Top count Microplate.

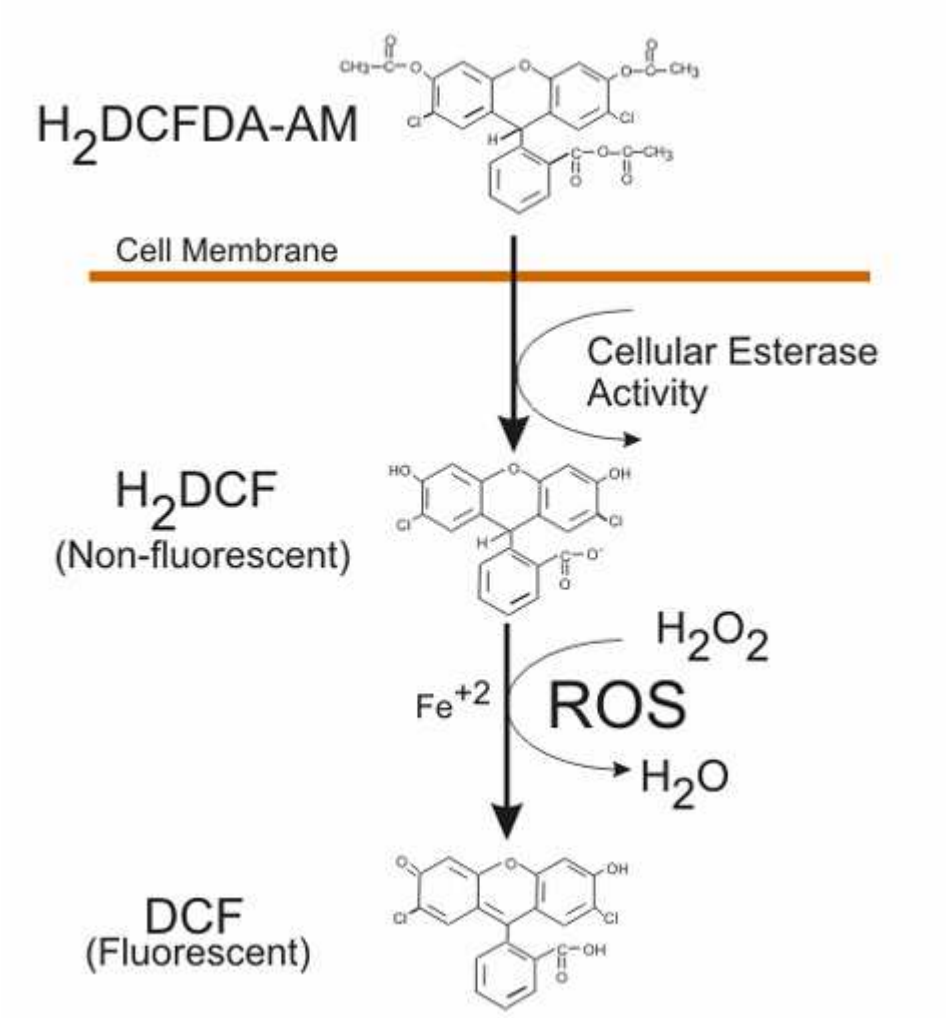

Figure 3.1: DCFH-DA assay for ROS detection.

3.5 NITRIC OXIDE SYNTHASE (NOS) ACTIVITY

NOS activity was measured in de-proteinated conditioned media of HUVEC or MEC by using the Griess method for nitrate quantification. This method requires NO_3^- first be reduced to NO_2^- and then determined by Griess reaction. The Griess reaction (Figure 3.2) is a two-step diazotization reaction in which the NO-derived nitrosating agent reacts with sulfanilic acid to produce a diazonium ion that is then coupled to N-1-naphthylethylenediamine to form a chromophoric azo product that absorbs at 540 nm. The concentrations of NO in the samples were determinated using a calibration curve generated with standard $NaNO_2$ solutions. Each experiment was performed in triplicate and repeated at least 3 times with similar results.

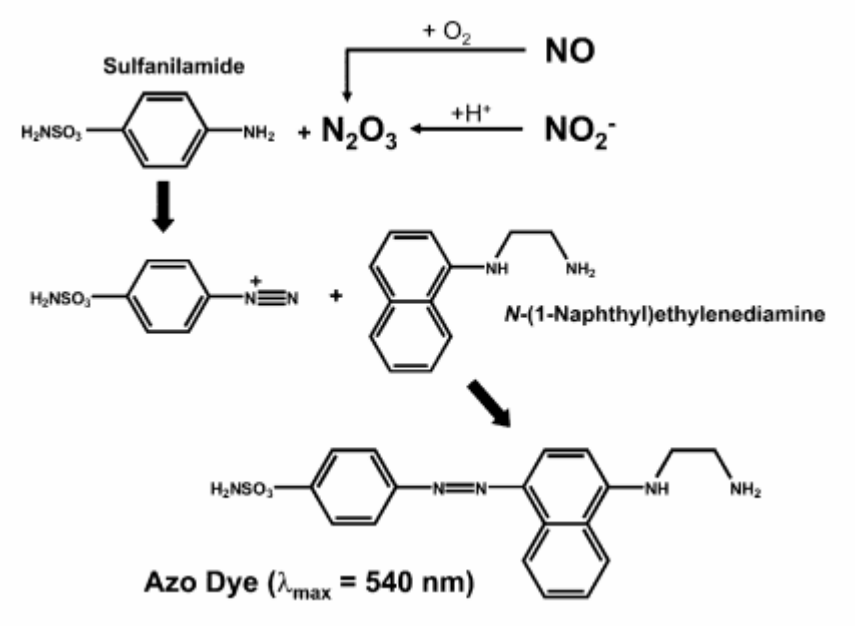

Figure 3.2: The Griess reaction.

The nitrosating agent dinitrogen trioxide (N_2O_3) generated from autoxidation of nitric oxide (NO) or from the acidification of nitrite (NO_2^-) reacts with sulfanilamide to yield a diazonium derivate. This reactive intermediate will interact with N-1-naphthylethylene diamine to yield a colored diazo product that absors strongly at 540 nm.

In collaboration with prof. Ciuffreda (University of the Study of Milan) we used a second method to measure NOS activity. After deproteinations of medium with acetone, the samples were spiked with the N-labelled nitrite and nitrate as internal standards to achieve final concentrations of 0.2 mM for N-nitrite and 2 M for N-nitrate. Derivatization of nitrite and nitrate

was performed using acetato di etile (Tsikas, 2000) and the measure was done with GC-MS analysis (GasCromatography mass spectrometry).

GC-MS was carried out on a Finningan Trace (DSQ) 7000 apparatus (San Jose, CA, USA) connected directly with Finnigan trace gas chromatograph.

3.6 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

A) NUCLEAR EXTRACTS

HUVEC were washed twice with cold PBS and centrifuged at 2500g for 10 min at 4°C. Cellular pellets were resuspended in 100µl of a solution A containig HEPES 10 mM pH 7.8, $MgCl_2$ 1.5 mM, KCl 10 mM, 1.4-Dithio-threitol (DTT) 2 mM and Phenylmethane-Sulfonyl fluoride 2 mM. After 10 minutes of incubation on ice 4µl of Triton 10X (v/v) were added and centrifuged at 13000 rpm for 1min at 4°C. To isolate only the nuclei, the nuclei-containing pellets were resuspended in a solution C containig HEPES 20 mM pH 7.9, $MgCl_2$ 1.5 mM, NaCl 420 mM, EDTA 0.2 mM, glycerol 25% and then incubated for 30 min at 4°C with constant shaking. Nuclear proteins were recovered in the supernatant after centrifugation at 13000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined with the Bradford reagent (Sigma Aldrich).

B) EMSAs

Single strand oligonucleotide with consensus binding-sites of NF-kB (Forward 5'-GGATCCTCAACAGAGGGGACTTTCCGAGGCCA-3') was labelled with T4 polynucleotide kinase (GE Healthcare, Milan, Italy) by using ^{32}P-ATP, annealed to the complementary strand (5-TGGCCTCGGAAAGTCCCCTCTGTTGAGGATCC-3'), and then purified by polyacrylamide gel electrophoresis. 5 µg of nuclear extract were incubated for 30 minutes at room temperature with 20 Kcpm of ^{32}P-labelled probe in binding buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 5% glycerol, 0.5 mM DTT). All binding reactions contained 0.5 µg poly (dI·dC) as a nonspecific competitor. For competition experiments, 50-fold excess of unlabelled specific or unrelated double-stranded sequences was added to the binding mixtures. For the gel supershift assay, nuclear extracts were incubated with antibodies (1 µg) against p65 and p50 (Santa Cruz Biotechnology-Tebu Bio, Magenta, Italy) for 60 min on ice followed by 25 min of incubation with oligonucleotide. Protein-DNA complexes were resolved on 5% acrylamide gels. Gels were dried and radiolabelled bands were detected by autoradiography. Oct-1 was used as a constitutive housekeeping transcription factor because of its apparently ubiquitous expression in mammalian cells (Veenstra et al. 1997). The experiments were repeated three times with similar results.

3.7 REPORTER GENE ASSAY

To study NFkB activity, subconfluente HUVEC and MEC were transfected with a luciferase reporter plasmid (Figure 3.3) (0.2 μg/cm^2) containing multiple copies of the NFkB consensus (pGL4.32[luc2P/NFkB-RE/Hygro]vector, Promega Italia, Milano) using Arrest-In Transfection Reagent (Invitrogen). Cells were cotransfected with the phRL-TK plasmid encoding *Renilla* luciferase (5ng/cm^2), as a control for differences in transfection efficiency. After 4h from transfection, the cells were exposed 24h to either in 0.1 and 1.0 mM Mg. Firefly and *Renilla* luciferase activities were measured using Dual-Luciferase Reporter Assay kit manual (Promega Italia, Milano). Results are shown as the mean ± standard deviation (SD) of three separate experiments in triplicate.

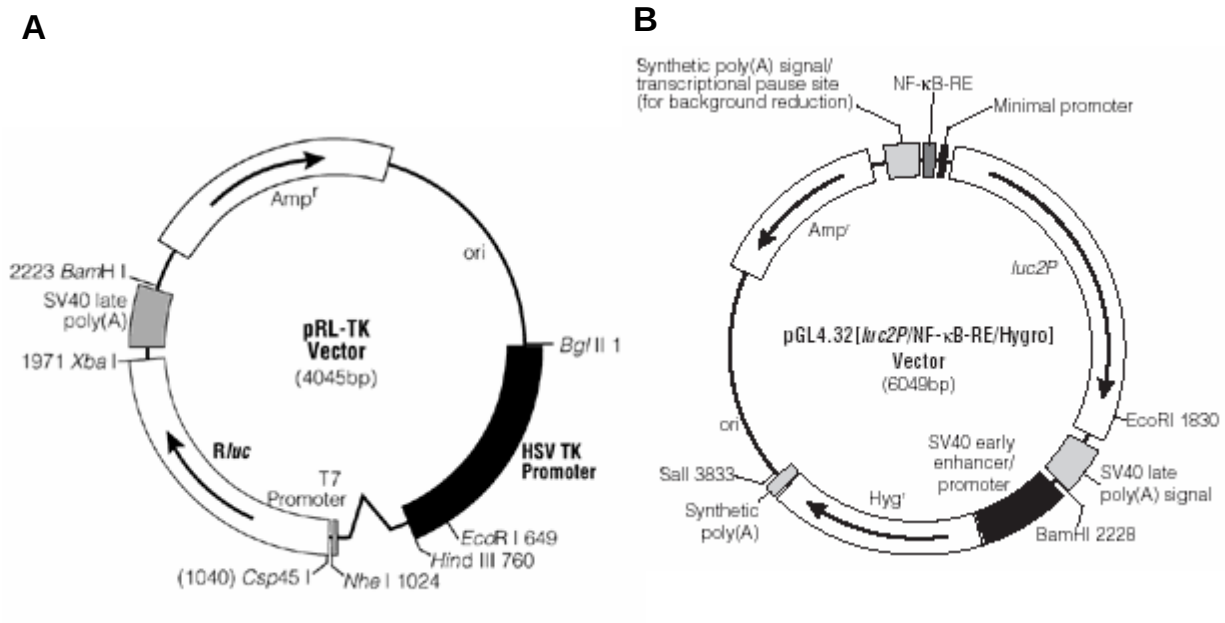

Figure 3.3: **A)** pRL-TK vector map and **B)** pGL4.32[luc2P/NFkB-RE/Hygro] vector**.**

The pGL4.32[luc2P/NFkB-RE/Hygro] vector contains five copies of an NFkB response element that drives transcription of the luciferase reporter gene *luc2P* (*Photinus pyralis*). Luc2P is a synthetically-derived luciferase sequence with humanized codon optimization that is designed for high expression and reduced anomalous transcription. The luc2P gene contains hPEST, a protein destabilization sequence. The vector contains an ampicillin resistance gene to allow selection in E.Coli and a mammalian selectable marker for hygromycin resistance.

3.8 REVERSE TRANSCRIPTION PCR

Total RNA was extracted from HUVEC cultured in 0.1mM, 1.0 or 5.0 mM Mg, wih TRIzol (Invitrogen) according to the manufacture's instructions. After quantification, 1μg of total RNA was reverse transcribed by Transcriptor First Strand cDNA Synthesis kit (Roche). The PCR amplification was carried out using 1/50 of the final RT reaction. Each amplification cycle consisted of :

T = 95°C for 30 second
T = 54°C for 60 second
T = 72°C for 60 second
T = 72°C for 5 minutes (last step)

The reaction was stopped after 30 cycles. One fifth of the reaction mix was separated on a 1% agarose gel. The sequence of TRPM7 and GAPDH primers synthesized by Primm srl (Milan, Italy) are the following:

TRPM7	**Sense: 5'-** CTTATGAAGAGGCAGGTCATGG **- 3'** **Antisense: 5'-** CATCTTGTCTGAAGGACTG **- 3'**
GAPDH	**Sense: 5'-** CCACCCATGGCAAATTCCATGGGA**- 3'** **Antisense: 5'-** TCTAGACGGCAGGTCAGGTCCACC**- 3'**

3.9 TransAM ASSAY

TransAM assays were performed according to the manufacture's instructions (Active Motif, Carlsbad, CA, USA). TransAM NFkB family kits designed specifically to quantify NFkB activation (Figure 3.4). In brief, 5 μg of nuclear protein samples was incubated for 1h in a 96-well plate coated with an oligonucleotide that contains the NFkB consensusn site (5'-GGGACTTTCC-3') to which activated NFkB homodimers and heterodimers contained in nuclear extracts specifically bind. By using an antibody directed against an epitope on p50 or p65 that is accessible only when NFkB is activated and bound to its target DNA, the NFkB complex bound to the oligonucleotide is detected. After incubation for 1h with a secondary horseradish peroxide-conjugated antibody, specific binding was detected by colorimetric estimation on a spectrophotometer at 450 nm. Results are expressed as arbitrary units and represent the mean ± SD of two separate experiments in triplicate.

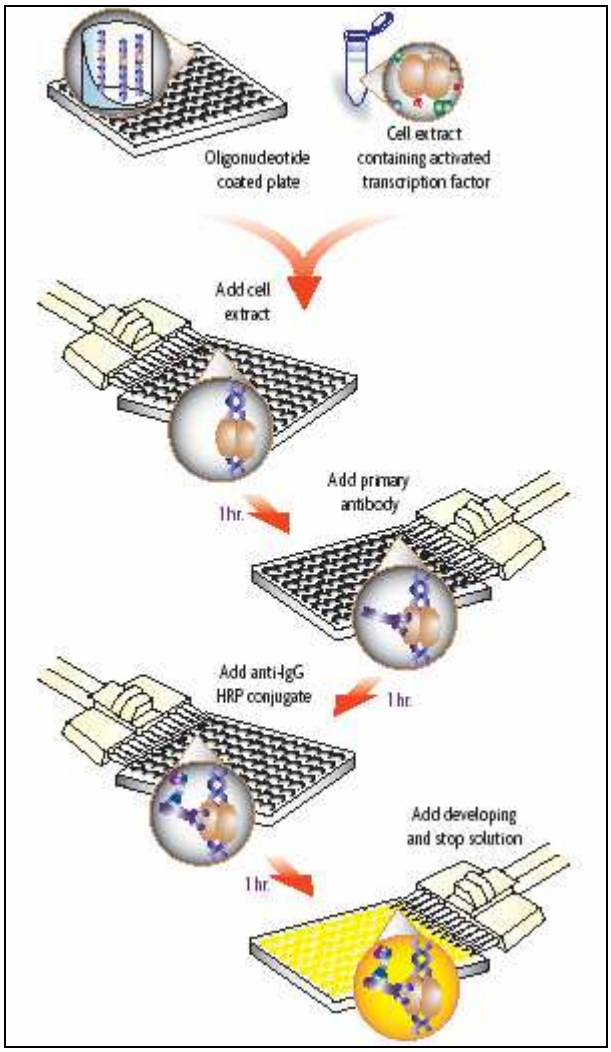

Figure 3.4: Flow chart of process.

3.10 PROTEIN ARRAY

HUVEC and MEC were cultured in medium containing 0.1 or 1.0 mM Mg for 2 days. 5 ml of conditioned media were centrifuged and utilized to incubate the membranes on which 40 antibodies against cytokines were spotted in duplicate (RayBiotech-Tebu Bio, Milano, Italy). The assay was performed according to the manufacturer's instructions (Figure 3.5). Densitometry was performed by the ImageJ software. Data are expressed as the mean ± standard deviation of two experiments in duplicate.

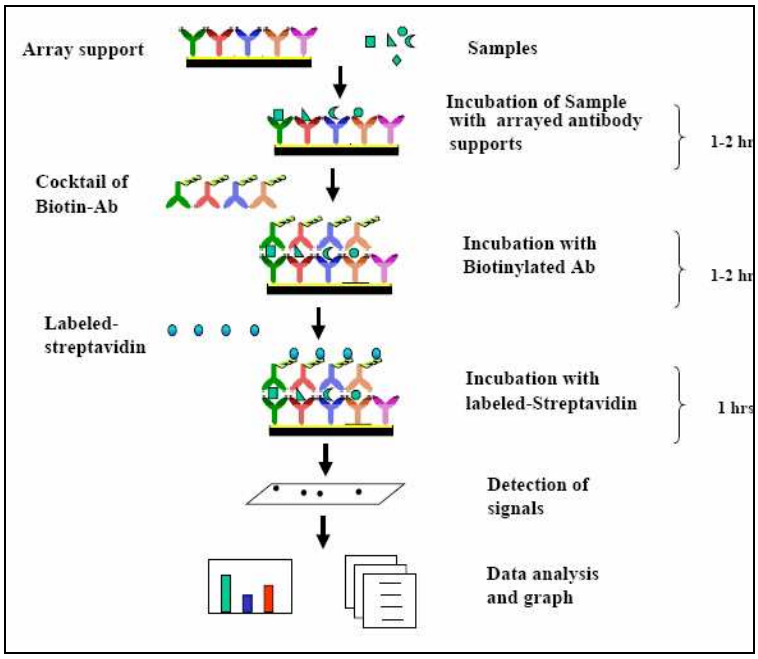

Figure 3.5: Representative scheme of protein array proces.

3.11 WESTERN BLOT ANALYSIS AND ELISA.

A) Culture media were resolved by SDS-PAGE, transferred to nitrocellulose sheets at 200 mA for 16 h, and probed with anti-PDGF-BB, anti-RANTES, anti-IL-6, anti-MMP-9 and anti-MMP-2 antibodies (Santa Cruz Biotechnology-Tebu Bio). Secondary antibodies were labeled with horseradish peroxidase (GE Healthcare). The SuperSignal chemiluminescence kit (Pierce, Rockford IL) was used to detect immunoreactive proteins. All the results shown were reproduced at least three times. Results are shown as the mean ± standard deviation.

B) Nuclear extracts were obtained using the Nuclear Extract kit (Active Motif) and proteins were separeted on 12.5% SDS-PAGE, transferred to nitrocellulose and processed by western blot using polyclonal antibodies against NFkB subunit (p65 or p50), actin, TATA binding protein (TBP) and glyceraldehyde-3-phosphate deydrogenase (GAPDH) antibodies (from Santa Cruz Tebu bio, Milan, Italy). Secondary anti-rabbit antibodies were labeled with horseradish peroxidase (GE Healthcare).

C) Cells (HUVEC or MEC) exposed at 0.1 or 1.0 mM Mg overnight, 24h or 48h were lysed in cold lysis buffer (10 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.5 mM EDTA and protease inhibitor cocktail (Sigma)) and centrifugated at 14000 rpm for 10 min at 4°C. Whole-cell extract were subjected to 8% SDS-PAGE and proteins were transferred to a nitrocellulose membrane sheets. Western blot analysis was performed using antibody anti-TRPM7 (Abcam), anti-p21, anti Cdc25B, anti-eNOS, anti-iNOS and anti-pSer1177 (from Cell Signaling and Santa Cruz Tebu bio). Secondary anti-rabbit or anti-goat antibodies were labeled with horseradish peroxidase (GE Healthcare).

The SuperSignal chemiluminescence kit (Pierce, Rockford, IL) was used to detect immunoreactive proteins.

The amounts of TIMP-2, IL-1α and IL-8 were measured in 1:2 diluted medium using a double-antibody sandwich ELISA (GE Healthcare) according to the manufacturer's instructions. The concentrations of TIMP-2, IL-1α and IL-8 were determined by interpolation from a standard curve and shown as the mean ± standard deviation.

Densitometric analysis was performed by the ImageJ software.

3.12 IMMUNOFLUORESCENCE

HUVEC and MEC were seeded on gelatin-coated coverslips, washed and fixed in PBS containing 3% paraformaldehyde and 2% sucrose (pH 7.6) for 10 min at room temperature. Cells were permeabilized with Hepes-Triton X-100 for 10 min and blocked with 2% bovine serum albumin (BSA) for 1h at room temperature. After washing cells were incubated with anti-p65 and p50 antibodies (Santa Cruz Biotechnology-Tebu Bio) for 60 min at 37°C and stained with rhodamine-labeled immunoglobulins against rabbit (Invitrogen). Coverslips were observed with a fluorescence microscopy (Olimpus AX70) at 40x-magnification. Staining with non immune IgGs did not yield any significant signal.

3.13 ZYMOGRAPHY ASSAY

Matrix metalloproteinase (MMP)-2 and MMP-9 activities were measured by gelatin zymography. Conditioned media were incubated at 4°C overnight with gelatin-sepharose. Zymography was performed using 8% polyacrylamide gels co-polymerized with 1 mg/ml gelatin type B (Sigma Aldrich) under nonreducing conditions without heating. Gels were then washed twice for 30 minutes in 2.5% Triton X-100 at room temperature and incubated overnight in collagenase buffer (50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $CaCl_2$, 150 mmol/L NaCl) at 37°C. Gels were stained in Coomassie Blue R250 (Bio-Rad, Milano, Italy) in a mixture of methanol: acetic acid: water (4:1:5) for 1 hour and destained in 10% acetic acid solution. Gelatinase activities (MMP-2 and MMP-9) were visualized as distinct white bands, indicating the proteolysis of the gelatin incorporated into the gel.

3.14 STATISTICAL ANALYSIS

All data were representative of at least three separate experiments. Densitometric analysis was performed by the ImageJ software on different blots and expressed using an arbitrary value scale. Results are shown as the mean ± standard deviation. Statistical significance was determined using the Student's t test and set at pvalues less than 0.05. In the figures, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.