The effect of magnesium on the inflammatory response in human vascular endothelial cells

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Declaration

I declare that the work presented in this thesis is my own work. And reference to other work has been cited accordingly with full details in the reference section. All studies in this thesis were performed under the supervision of Professor Simon Langley-Evans, Department of Nutritional Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, UK, LE12 5RD

Lujain Almousa

Dedicated

To my grandma, my parents, my husband, my kids and to all my siblings with love and appreciation

ABSTRACT

Magnesium plays several physiological roles, including the formation of bones and teeth, muscle contraction, and cardiovascular function. Magnesium deficiency affects cardiovascular health through the modulation of endothelial cell function, particularly by having a negative impact on endothelial cell proliferation, increasing monocyte adhesion, inhibiting cell migration and markedly altering endothelial cell gene expression. With the recognition of the protective role of magnesium in endothelial cells, the aim of this study was to investigate the effects of magnesium sulphate on human umbilical vein endothelial cells (HUVECs) proliferation, gene expression, and the pro-inflammatory response caused by a bacterial endotoxin lipopolysaccharide (LPS).

After culture for 120 hours in low (0.1mM) and high (5mM) magnesium, the viability of HUVECs decreased in the low magnesium (P=0.024), whilst the proliferation of HUVECs increased in the high magnesium (P=0.016). Moreover, exposing the cells to LPS lowered viability in the culture with low magnesium (P=0.002), but high magnesium protected the HUVECs from LPS-induced cell death (P=0.037).

Magnesium dose-dependent gene expression profiles of HUVECs were investigated via microarray, revealing extensive effects of low and high magnesium on the transcriptome (2930 up-regulated genes and 2828 down-regulated genes).

Magnesium deficiency in endothelial cells activated inflammatory pathways through the transcription factor nuclear factor κB (NF-κB), which plays an important role in the early pathology of atherosclerosis. LPS-treated HUVECs cultured in low magnesium showed up-regulation of mRNA expression for pro-inflammatory factors, such as ICAM-1, VCAM-1, IL-8, and MCP-1, and the expression of cytokine proteins, including IL-2, IL-3, IL-8, IL-15, MCP-1, GRO, and GROa. In contrast, high magnesium decreased the expression of IL-6 and MCP-1 mRNA and the protein concentrations of IL-2 and IL-6. The study found that LPS activates the NF-κB pathway through the TLR2 and TLR4 receptors and this pathway was inhibited by high magnesium concentration.

Overall, the work presented demonstrates that inflammatory processes in vascular endothelial cells are sensitive to magnesium. This suggests that magnesium nutrition may be an overlooked factor in CVD and is worthy of further investigation.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BH4	Tetrahydrobiopterin
Ca^{2+}	Calcium ions
CaM	Calmodulin
CAMs	Cell adhesion molecules
cAMP	Cyclic adenosine monophosphate
CRP	C-reactive protein
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGM-2	Endothelial cell growth medium
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
FMD	Flow-mediated vasodilation
FMN	Flavin mononucleotide
GLUT4	Insulin-regulated glucose transporter
GM-CSF	Granulocyte macrophage-colony stimulating factors
GRO	C-X-C Motif Chemokine 1
HDL	High-density lipoprotein
Helicase XPD	xeroderma pigmentosum group D helicase
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular cell adhesion molecule-1
IFN	Interferons
Ig	Immunoglobulin
IκB	NF-κB inhibiter

Ikk	I kappa Kinase
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-12	Interleukin-12
iNOS	Inducible NOS
IRAK-4	IL-1 receptor-associated kinase-4
LCAT	Lecithin cholesterol acyl transferase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRNI	Lower reference nutrient intake
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
mg/d	Milligram per day
mg/dl	Milligrams per Deciliter
mg/L	Milligram per Litre
Mg^{2+}	Magnesium ions
MgCO3	Magnesium carbonate
$MgSO_4$	Magnesium sulphate
mmol/L	Millimoles per Litre
MMR	Mismatch repair
MTT	Tetrazolium salt (3-(4,5-dimethylthiazol)-2,5 diphenyl tetrazolium bromide)
MutL	Methyl-directed mismatch repair protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NER	Nucleotide excision repair

NF-κB	Transcription factors of the nuclear factor–kappa B
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
Nuclease XPG	Xeroderma Pigmentosum Complementation Group G (XPG) protein
O_2	Oxygen
Ox-LDL	Oxidative LDL
PBS	Phosphate buffered saline
PGI2	Prostacyclin
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SFM	Magnesium-free medium
TF	Tissue factor
TG	Triglycerides
THP-1	Human monocytic cells
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
TRAF6	TNF receptor-associated factor 6
TRP	Transient receptor potential
TRPM6	Transient receptor potential melastatin-6
TRPM7	Transient receptor potential melastatin-7
VCAM-1	Vascular cell adhesion molecule-1

1

INTRODUCTION

1 Introduction

Disorders related to poor magnesium status are a common problem worldwide (Chakraborti et al., 2002). The National Diet and Nutrition Survey in the United Kingdom reported that approximately 10% of men and 9% of women, aged 19 to 64, consumed less than the lower reference nutrient intake (LRNI), which is often an indicator of widespread deficiency in the population. In some population subgroups intakes are particularly low, with 27% of adolescent boys and 48% of adolescent girls consuming below the LRNI for magnesium (Whitton et al., 2011). In Germany, a cross-sectional study found that 15% of the subjects had low serum magnesium concentrations (Vormann, 2003). Additionally, in the United States, the dietary intake of magnesium is inadequate. The mean daily intake in the US is 288 mg, considerably less than the recommended daily allowance of 310 mg for women and 400 mg for men (King et al., 2009). Magnesium status is not just related to intake, there can be acute losses of magnesium from the body due to malabsorption diarrhoea, vomiting, increase renal losses and alcoholism (Fawcett et al., 1999). Large intakes of magnesium from food sources are not harmful, but large amounts of magnesium salt can have a cathartic effect (Mann and Truswell, 1998).

Magnesium is an important cation for normal health and physiological functions. It is a cofactor in numerous metabolic processes. Additionally, magnesium is essential in the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Furthermore, the functions of more than 300 enzymes depend on the presence of magnesium (Swaminathan, 2003).

Various studies have suggested an association between chronically low magnesium levels and an increased risk of cardiovascular disease (CVD) (Cunha et al., 2012). Moreover, many studies have focused on the role of magnesium in heart health. Findings from these studies provide evidence that poor magnesium nutrition may adversely affect the cardiovascular system by altering the risk of various cardiometabolic outcomes (Champagne, 2008, Fox et al., 2004, Swaminathan, 2003). Magnesium impacts on several

mechanisms that affect cardiovascular disease (Houston, 2011), which encompasses diseases such as peripheral vascular disease and coronary artery disease (Lüscher, 2016).

CVD is a major cause of death worldwide and accounts for approximately 31.5% of all deaths. In the United Kingdom, CVD is responsible for approximately 180,000 deaths each year, making CVD the primary cause of death in the country, and costing the National Health Service (NHS) approximately 19 billion pounds per annum (Townsend et al., 2012). CVD deaths are associated with increased CVD risk factors, including hypertension, diabetes, obesity, increased aging and poor diets.

1.1 The Vascular System

1.1.1 Blood Vessels

Blood vessels are networks of tubes that convey blood from the heart toward all organs and tissues and then return it to the heart. This closed system, called the circulatory system, is subdivided into two main types: systemic arteries and the venous system. The systemic arteries carry nutrient-rich blood (oxygen, water, glucose, fatty acids, etc.) to the tissues and organs of the body. The arterial walls in systemic arteries have smooth muscle cells and collagen fibres, which help in accommodating high pressure blood flow in these vessels. The venous system transports waste products (carbon dioxide, creatinine, urea, etc.) from the organs. The venous walls have fewer elastic fibres, so they are thinner, allowing large amounts of blood to return to the heart. All arteries branch to make a smaller network of capillaries, some of which have a diameter of less than 100μm, making them microscopic. Capillaries have thin walls comprised of one layer of endothelial cells that eases the transport of substances between the blood and tissue. The capillaries also have slower blood flow and lower blood pressure (Little and Little, 1989).

1.1.2 Great Vessels

The "great vessels" are the main vessels that directly join the heart. The great veins are the inferior venae cava, the pulmonary veins and the superior venae cava. The great arteries are the aorta and the pulmonary trunk (Russ, 2006). The inferior venae cava is the vein that collects blood from the lower part of the body and returns it to the heart. Alternatively, the superior vena cava collects blood from the upper part of the body. The pulmonary veins and arteries, a part of pulmonary circulation, move blood between the heart and the lungs to resupply the blood with oxygen and remove carbon dioxide (Sherwood, 2012).

The aorta is the largest artery in the body. It carries oxygenated blood from the left ventricle of the heart through the chest and abdomen. To be able to compensate for the heart's contractions, the aorta has more fibres to facilitate its expansion. It divides into smaller arteries that supply all areas of the body. Both the left and right coronary arteries, which supply the heart muscle, divide off from the aorta (Little and Little, 1989).

1.1.3 Vascular Structure

Arterial walls have three layers: The tunica externa is the tough outer layer with fibres and collagen that constitute the connective tissue; the tunica media is a thicker layer of smooth muscle in the middle of the artery wall; and the tunica intima consists of a membrane and smooth tissue called the endothelium (Figure 1.1) (Zhao et al., 2015).

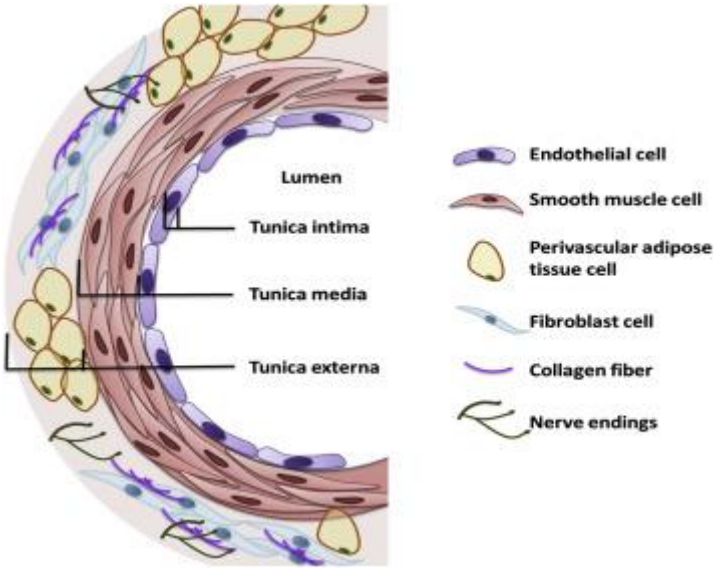


Figure 1.1 Blood vessels' three-layered structure (Zhao et al., 2015).

1.1.4 Vascular Endothelium

The endothelium is a monolayer of endothelial cells covering a surface area of over 1,000m^2 for all vascular tissue in adults. The dimensions of an endothelial cell are up to 5 μm of depth , with a width of 10–15 μm and a length of approximately 25–50 μm (Fitridge and Thompson, 2007). This single layer has an intercellular margin between some of the cells that serves as a pathway to interchange small substances and fluid from the vessel walls (Little and Little, 1989). The endothelium plays a critical role in the circulation and maintenance of the artery wall's health and balance by releasing various vasodilators and vasoconstrictors, such as nitric oxide (NO), prostacyclin (PGI_2), endothelin-1 (ET-1) (Schiffrin, 2001), angiotensin I, angiotensin II and platelet activating factor (Noble, 2002). These vasoactive molecules manage the vascular tone, which then provides the metabolic requirements and oxygen quantities that the tissues need (Deanfield et al., 2007).

The endothelium protects the artery by regulating the efficiency of the immune and inflammatory responses. It also assists in controlling the blood fluidity by regulating platelet adherence, thrombosis and thrombolysis (Sumpio et al., 2002). The structure of the endothelium as a basement membrane serves to control vessel wall permeability by regulating the transfer and circulation of particles between the blood and others layers (Stevens et al., 2000). Any disorder in endothelial activity may result in development of cardiovascular diseases risk factors (Sumpio et al., 2002), including hypertension, thrombosis and atherosclerosis (Schiffrin, 2001, Cines et al., 1998).

1.1.5 Cardiovascular Disease

CVD is a major cause of death worldwide. CVDs include coronary artery disease, cerebrovascular disease and disorders of the peripheral vascular system. The underlying chronic cause of these diseases is atherosclerosis, while thrombosis, resulting in blockage of an artery causes acute events such as a myocardial infarction (heart attack) or stroke. Alternatively, atherosclerosis may weaken the artery wall, resulting in an aneurysm and potential rupture of the artery (Stanner, 2005).

1.1.5.1 Types of Cardiovascular Disease

There are many different types of CVD. When atherosclerosis affects the heart's arteries, it is referred to as coronary artery disease, the most common type; causing myocardial ischaemia. Congestive heart failure is defined as cardiac dysfunction, where the heart can't pump the required amount of blood and oxygen to the body's tissues. Peripheral vascular disease involves damage to the peripheral arteries (the blood vessels distant from the heart), primarily the large and medium arteries that supply the limbs, usually the legs (Edward *et al.,* 1999). Cerebrovascular disease is caused by disruption to the arteries that supply the brain, commonly resulting in a stroke and leading to damage to the brain tissue (Stanner, 2005).

1.1.5.2 Risk Factors for Cardiovascular Disease

Several factors are associated with an increased risk of CVD, including hypertension, obesity, high blood cholesterol levels, diabetes mellitus, smoking and physical inactivity (Stanner, 2005). Hypertension causes endothelial damage, leading to atherogenesis and

CVD. With time, hyperglycaemia associated with diabetes mellitus damages the large arteries and causes microvascular complications as a result of endothelial damage and increased low-density lipoprotein (LDL) levels. Nicotine in tobacco reduces the oxygen-carrying capacity of the blood, ultimately increasing the risk of myocardial infarction and cardiac ischaemia (Aaronson and Ward, 2007). Obesity is associated with a range of metabolic diseases including type-2 diabetes, atherosclerosis and hypertension. In obesity, intracellular lipids increase and effect the functions of tissues. Physical activity and healthier daily living activities enhance overall health and improve a number of CVD-related risk factors, such as insulin resistance, glucose tolerance, blood pressure, body weight and blood lipid levels (Stanner, 2005). Hypercholesterolemia is also associated with atherosclerosis due to the endothelial dysfunction that it causes and the deposition of modified cholesterol, leading to atherosclerosis, and associated injury to the endothelial cells (Vogel, 1997).

1.1.5.3 Atherosclerosis

As described, the endothelial cells provide an essential layer that lines the interior surface of the blood vessels, playing a critical role in maintaining blood flow and in the movement of molecules from the bloodstream to the tissues. Damage or injury to this layer can promote the formation of an atherosclerotic plaque (van Hinsbergh, 2012). Atherosclerotic plaques represent the accumulation of lipid, fibrous tissue and smooth muscle cells within the vessel wall (Gibney *et al.*, 2005), which, in turn, leads to atherosclerosis (Szmitko *et al.,* 2003).

Hypercholesterolemia and hypertension can have major effects on endothelial function, as they can result in increased ET-1, inflammatory responses and decreased NO production. In response to this, the endothelial cells secrete adhesion molecules and inflammatory cytokines which contribute to the recruitment and attachment of monocytes to the cell surface (Szmitko *et al.,* 2003). Vascular cell adhesion molecule-1 (VCAM-1) is the first adhesion molecule present in the endothelial surface layer and binds to the monocytes. Thereafter, the cell releases monocyte chemoattractant protein-1 (MCP-1) to help with the migration of the monocytes from the tunica intima to the subendothelial region. The

endothelial cells secrete macrophage colony-stimulating factor (M-CSF), which drives the differentiation of the subendothelial monocytes to macrophages (Maier, 2003). Endothelial dysfunction increases permeability, so that material from the bloodstream, including LDL moves into the subendothelial space. Within the artery wall, LDL is prone to oxidation and becomes oxidized LDL (ox-LDL), a key influential factor in plaque pathogenesis (Kaperonis *et al.,* 2006). Once the monocytes have migrated into the subendothelial space and have differentiated into macrophages, they take up ox-LDL via their scavenger receptors, breakdown the particles and store the cholesterol released. These macrophages accumulate large amounts of cholesterol ester in lipid droplets and develop into 'foam cells' (Hansson *et al.,* 2006). The excessive lipid accumulation may drive the foam cells to form fatty streaks on the artery wall and when they die they release cholesterol ester into the extracellular matrix, enhancing the inflammatory signalling (Moore et al., 2013). Endothelial cells also increase the production of platelet-derived growth factor (PDGF), which drives the migratory process and proliferates smooth muscle in the subendothelial space (Figure 1.2) (Maier, 2003).

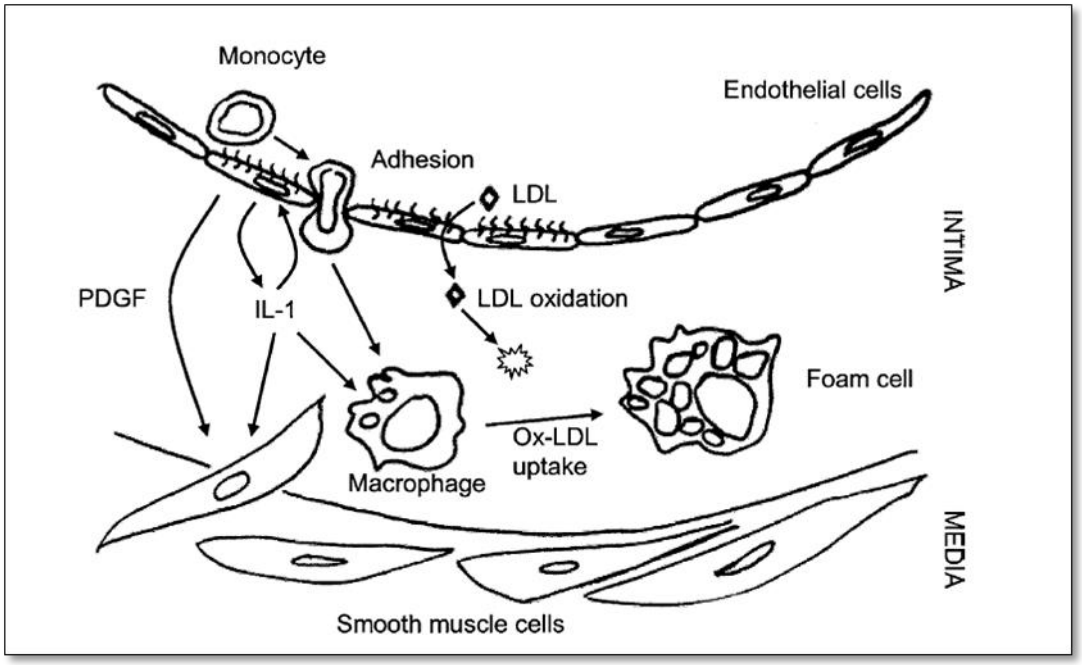


Figure 1.2 The role of endothelial cell dysfunction in atherosclerosis (Maier, 2003)

1.1.5.4 Micronutrients and Cardiovascular Disease Risk

The consumption of antioxidant vitamins (A, C and E) and lycopene has been demonstrated to lower CVD risk. These antioxidant protect LDL from oxidation through the action of free radicals (Clarke and Armitage, 2002). Vitamins B_6, B_{12} and folate are needed for homocysteine metabolism. Elevated levels of homocysteine in the blood are a risk factor for coronary heart disease because they correlate with inflammation, oxidative stress, cholesterol synthesis and blood clotting (Rolfes *et al.,* 2015). An insufficient intake of copper is also associated ischaemic heart disease, with the implication that copper is a modifier of lipid metabolism. In addition, a selenium deficiency causes Keshan disease, a congestive cardiomyopathy (Bogden and Klevay, 2000). Zinc is essential to many antioxidant enzymes in helping to protect the endothelial cells from inflammation and apoptosis. Insufficient serum zinc levels have also been linked to heart failure (Gibney *et al.,* 2005). As described below, magnesium has also been linked to CVD through a variety of mechanisms

1.2 Control of Vascular Tone

Vascular tone is the tension exerted by the vascular smooth muscle cells in the artery walls to maintain the blood flow (Jackson, 2000). Increasing the vessel tone results in vasoconstriction and decreased blood flow. In contrast, the reduction in the vessel tone causes vasodilation and increased blood flow (Levick, 2010). Endothelial cells have an important role in controlling vascular tone, by releasing and responding to many vasoactive molecules that regulate the constriction and the relaxation of the vessels (Deanfield et al., 2007). NO and PGI2 are vasodilators, while ET-1 is a vasoconstrictor (Aaronson and Ward, 2007).

1.2.1 Nitric Oxide

Nitric oxide synthase (NOS) is an enzyme that produces the gaseous radical NO. This enzyme has three isoforms: endothelial NOS (eNOS), which is expressed in endothelial cells; inducible NOS (iNOS), which is expressed by macrophages; and neuronal NOS (nNOS), expressed in neurons and skeletal muscle. All of them generate nitric oxide by the same mechanism; the oxidation of L-arginine. The local concentration of calcium ions

(Ca^{2+}) regulates the activity of eNOS and nNOS, while macrophages activate iNOS expression (Feng, 2012, Liu and Huang, 2008).

In endothelial cells, NO is produced via the metabolism of L-arginine by eNOS, which is activate by Ca^{2+} and calmodulin (Ca^{2+}/CaM) (Andrew and Mayer, 1999, Marletta, 1993). Binding Ca^{2+}/CaM to their binding site allows the electron donated by nicotinamide adenine dinucleotide phosphate (NADPH) to transfer to the oxygenase domain to activate the synthesis of NO (Alderton et al., 2001). NO has a very short half-life (Haenni et al., 2002), and diffuses to the vascular smooth muscle, resulting in the relaxation of this muscle. This is an important step in controlling the vessel tone (Kietadisorn et al., 2012).

eNOS has an oxygenase domain, which is the area where NO is formed by catalysing conversion of L- arginine to L-citrulline in the presence of oxygen O_2. Tetrahydrobiopterin (BH4) acts as a cofactor while NADPH transfers the electron through the flavin adenine dinucleotide (FAD)–flavin mononucleotide (FMN) axis. Ca^{2+} -calmodulin is essential in linking FMN and heme iron to ensure efficient electron transfer by facilitating the FMN donation of electrons and the alignment of the FMN and heme domains (Figure 1.3) (Zhang et al., 2014).

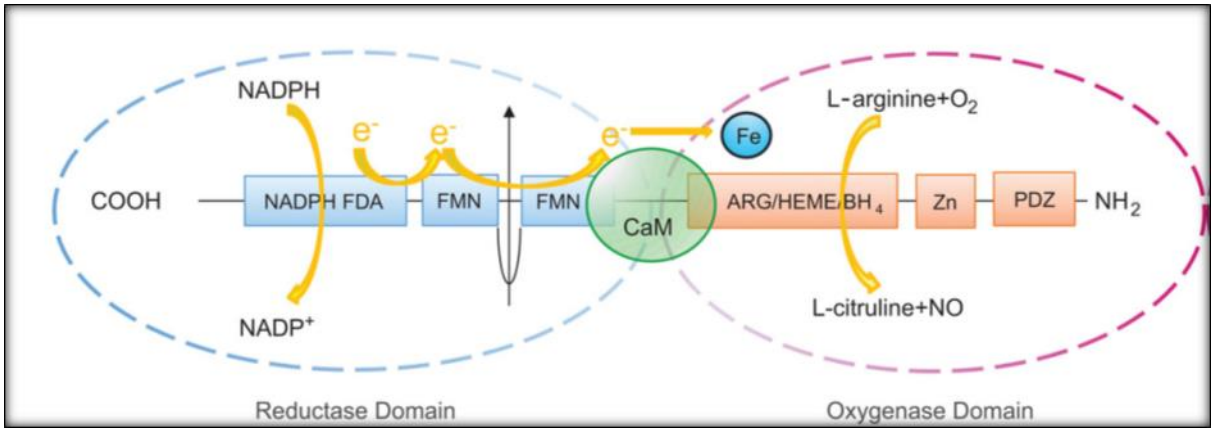


Figure 1.3 Structure of the nitric oxide synthase protein (Zhang et al., 2014).

A decrease in the expression of eNOS is associated with several cardiovascular risk factors due to the important role of NO in protecting endothelial cells, such as controlling vessel

tone, the proliferation of smooth muscle cells and platelet aggregation, and inhibiting leukocyte adhesion (Förstermann and Münzel, 2006).

1.2.2 Endothelins

Endothelins (ETs) are a group of three short peptides (ET-1, ET-2 and ET-3) that consist of 21 amino acids, secreted by the brain, kidneys and endothelial cells. ET-1 is produced by the vascular endothelium as a vasoconstrictor and raises the blood pressure. Many stimuli can increase the production of ET-1, such as lipoproteins, free radicals and stress. The ET-1 receptor, ET_A is located on vascular smooth muscle cells, and constriction of the smooth muscle cells results from activation of ET_A receptor by ET-1 (Agapitov and Haynes, 2002). Binding of ET-1 to its receptor ET_A increases calcium release, which affects water and salt balance and makes the vessels contract, increasing vascular tone. In addition, NO can inhibit ET-1 actions (Laurant and Berthelot, 1996).

1.3 The Transcription Factors of the Nuclear Factor–kappa B Family of Transcription Factors and the Link between Inflammation an Atherosclerosis

1.3.1 Nuclear Factors NF-κB

The transcription factors of the nuclear factor–kappa B (NF-κB) Rel family include many members, such as NF-κB 1 (p50/p105), NF-κB 2 (p52/p100), p65 (RelA), RelB and RelC (Tak and Firestein, 2001). They exist in the cytoplasm of most cells and play an important role in the regulation of inflammatory responses (Lindström and Bennett, 2005), including regulation of the expression of adhesion molecules and cytokines which are related to many critical diseases. In the cytoplasm, NF-κB exists in an inactive form. Phosphorylation of IκB, a regulatory protein that inhibits NF-κB activity, degrades the IκB-p65-p50 complex, resulting in release of activated NF-κB subunits p50 and p65, which then translocate to the nucleus (Sun and Andersson, 2002, Valen et al., 2001). In the nucleus, these active components of NF-κB binds to the κB enhancer elements of target genes. This

results in the transcription of many pro-inflammatory genes (Figure 1.4) (Tak and Firestein, 2001).

Under normal circumstances endothelial cells produce IκB, which inhibits NF-κB subunit activity, but in endothelial dysfunction, NF-κB is activated via the degradation of IκB by proteasomes, generally in response to inflammatory signals. These inflammatory signals include tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and lipopolysaccharide (LPS). Additionally, oxLDL and oxidative stress can modulate NF-κB activation (Collins et al., 1995).

The activation of NF-κB plays a key role in atherosclerosis by regulating several genes that play a mechanistic role in the development of atherosclerotic plaques, such as IL-1, TNF-α, tissue factor (TF), intercellular cell adhesion molecule-1 (ICAM-1), VCAM-1, M-CSF, MCP-1 and granulocyte macrophage-colony stimulating factors (GM-CSF). In smooth muscle, NF-κB activation up-regulates M-CSF and GM-CSF, which increase the proliferation of smooth muscle cells (Brand et al., 1996).

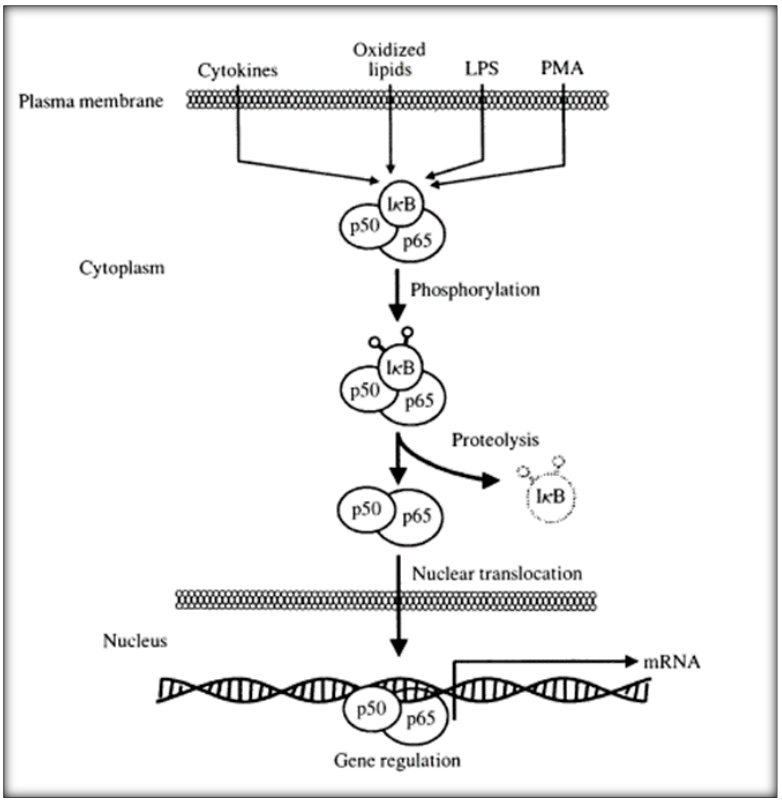


Figure 1.4 Activation of NF-κB (Brand et al., 1997).

1.3.2 Cytokines

Cytokines are groups of small proteins released by many different kinds of cells in all tissues to act within tissues to regulate the immune system (Hopkins, 2003). The main sources of pro-inflammatory cytokines are macrophages and monocytes, but these agents are also released by non-immune cells, including endothelial cells and fibroblasts in response to tissue injury (Murtaugh et al., 1996, Barrett, 1996). The cells in the layers of the artery wall are both a target and source of pro-inflammatory cytokines (Sprague and Khalil, 2009). Moreover, the main inducer of pro-inflammatory cytokine production is LPS from gram negative bacterial cell well (Murtaugh et al., 1996). In endothelial cells, binding LPS to its binding site toll-like receptors (TLR) responsible for activating NF-κB (Peters et al., 2003). Cytokines include many sub-families: interleukins, chemokines, tumour necrosis factors, and interferons (Hopkins, 2003). These categories include many branches, for instance: interleukins (e.g. IL-1, interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin6 (IL-6) and interleukin-12 (IL-12)), tumour necrosis factors (TNF-α and TNFβ), interferons (IFN-α, β and γ) (Sprague and Khalil, 2009) and chemokines (interleukin-8 (IL-8), C-X-C Motif Chemokine 1 (GRO), GROα, and MCP-1) (Murdoch and Finn, 2000).

IL-1 is an interleukin found at low levels in endothelial cells during normal physiological homeostasis. In contrast, the enhanced release of IL-1 results in release of IL-6, IL-8, MCP-1, GM-CSF and M-CSF, thereby triggering an inflammatory cascade (Carvalho and Savage, 1997). Another important cytokine in mediation of inflammation is TNF-α, which is released from activated macrophages and causes cell damage. TNF-α activates cells through the NF-κB signalling pathway (Peters et al., 2003) and causes apoptosis in many types of cells through a variety of mechanisms (Sprague and Khalil, 2009, Wajant et al., 2003). Additionally, IL-6 is an interleukin that stimulates B cells differentiation to secrete antibodies (Hunter and Jones, 2015).

IL-8 is one of the main chemokines involved in the attraction of neutrophils to inflammatory sites (Murtaugh et al., 1996). Likewise, MCP-1 is a chemokine involved in

the chemotaxis of monocytes, and it appears in atherosclerotic lesions (Zernecke and Weber, 2005).

The activation of the cell surface TLR in response to infection activates NF-κB, which induces the release of the early cytokines, such as TNF-α, IL-1 and IL-6. These pro-inflammatory cytokines (TNF-α and IL-1) stimulate the same target cells. This activation results in the production of adhesion molecules and the other cytokines (Figure 1.5) (Hopkins, 2003), and in this way infection and other inflammatory events disrupt vascular endothelial cell function and initiate the pathway potentially leading to atherosclerosis.

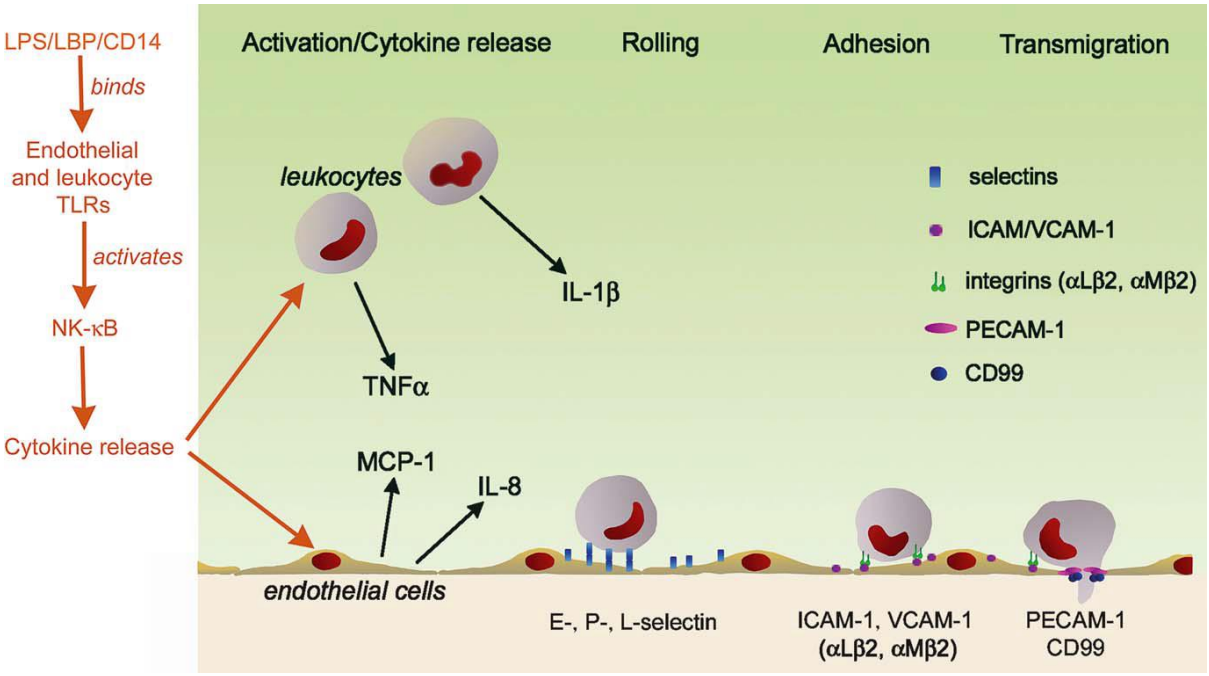


Figure 1.5 Stimulation endothelial cells by LPS and the result of TLR receptor activation (Peters et al., 2003).

1.3.3 Adhesion Molecules

Cell adhesion molecules (CAMs) are cell surface proteins that play a role in inflammatory responses by binding leukocytes to endothelial surfaces and transferring them to the site of inflammation (Piconi et al., 2004). As a response to inflammation, endothelial cells express leukocyte ligands ICAM-1 and VCAM-1 which are immunoglobulin (Ig)-like cell adhesion molecules. Binding to these adhesion molecules allows transmigration of leukocytes from the blood vessel lumen, through the barrier of endothelial cells and into the tissue underneath it (McHale et al., 1999). ICAM-1 is a transmembrane glycoprotein

that has five extracellular Ig-like domains and a cytoplasmic tail (Vainer, 2005), while VACM-1 has two forms: either six or seven Ig-like domains (Cook-Mills et al., 2011). NF-κB and cytokines such as TNF and IL-1 control the expression of ICAM-1 (Xu and Li, 2009), and VCAM-1 (Cook-Mills et al., 2011). Increased expression of ICAM-1 and VCAM-1 enables monocyte migration through the endothelium and their subsequent differentiation to macrophages which will accumulate oxidized low density lipoprotein to become foam cells (Cybulsky et al., 2001).

The mechanisms of leukocyte migration into the sub-endothelial space are mediated by cell adhesion molecules and can be expressed in four steps: 1) the rolling of leukocytes along the top side of the endothelium and settling due to selectins; 2) the activation of the leukocyte surfaces by local factors produced by the endothelium, increasing adhesion to the surface, which is mediated by chemokines; 3) adhesion to the endothelial surface; and 4) the leukocytes' transmigration across the endothelial barrier to the sub-endothelial space (Figure 1.6) (Collins et al., 1995).

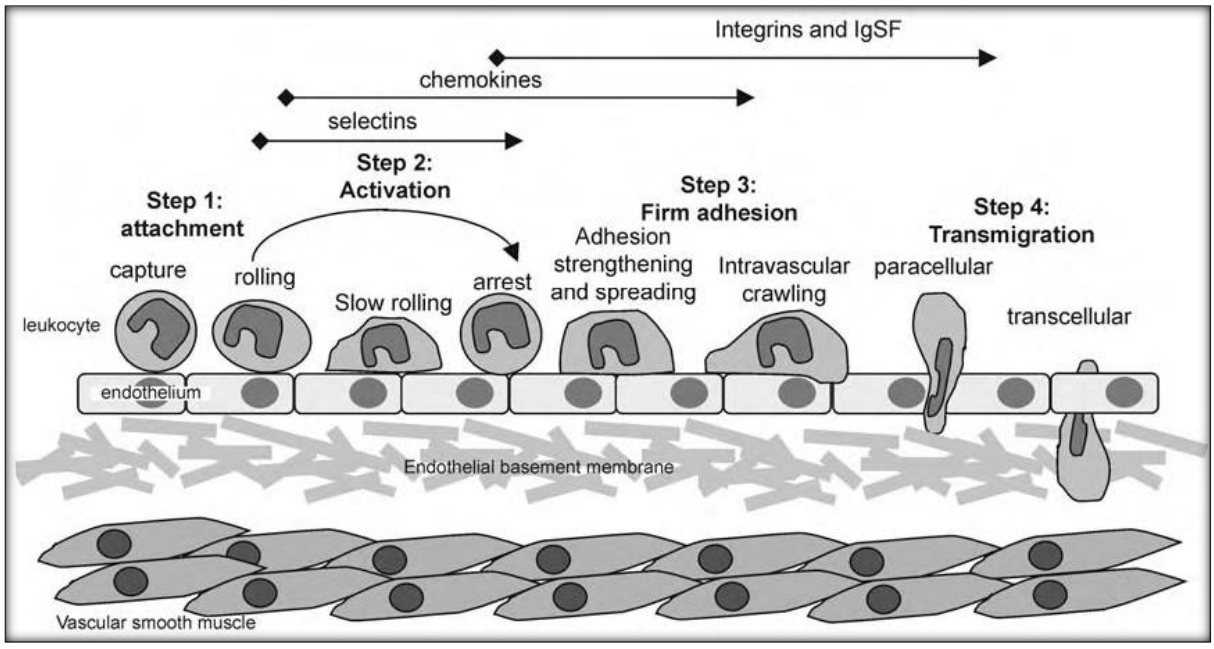


Figure 1.6 Migration of leukocytes into the sub-endothelial space (Lawson and Wolf, 2009).

1.4 Magnesium

Magnesium is a chemical element, specifically an alkaline earth metal that is silvery white and very lightweight. While it is one of the most common elements in the earth's crust, it is never found free in nature (Jahnen-Dechent and Ketteler, 2012) with the most common naturally occurring forms being magnesium carbonate ($MgCO_3$) and magnesium sulphate ($MgSO_4$) (Maguire and Cowan, 2002).

Magnesium was first discovered in 1808 by English Chemist, Sir Humphrey Davy, who isolated many elements by using electrolysis. Magnesium is named for magnesia, a region in Greece where magnesium carbonate was found. For many decades after Davy's discovery, magnesium was used only in industry in some European countries and in America. In 1878, magnesium started being used in clinical cases. A physician named Edward C. Huse operated on three patients using ligatures made from magnesium wire to prevent bleeding. Huse noticed that magnesium wires corroded more slowly. Erwin Payr added significant work to Huse's findings and developed surgical methods using magnesium implants in various parts of the body (Witte, 2015).

Magnesium deficiency was first identified in 1932 when McCollum fed rats a low magnesium diet. He noticed these rats had neuromuscular irritability and hyperaemia. These early experiments identified magnesium as an important mineral with many functions in the body, including bone function, parathyroid hormone secretion, vitamin D metabolism and as second messengers (Mann and Truswell, 1998) .

1.4.1 Food Sources and Recommended Intakes

Magnesium is ubiquitous in foods by virtue of its natural abundance in soils and uptake by plants. The best sources of magnesium are whole grain cereals, green leafy vegetables, legumes and nuts, whereas calcium-rich dairy products are poor in magnesium. Intermediate magnesium sources include meats, fish, fruits, vegetables and chocolate (Table 1.1). One of the most significant sources of magnesium for humans comes from 'hard' drinking water, which contains approximately 30 mg/L of magnesium (Swaminathan, 2003), while the 'soft' water content is 10 mg/L magnesium (Rolfes et al.,

2015). In the United Kingdom, the main source of magnesium in the diet is bread, breakfast cereals, potatoes, fish and beer (Henderson et al., 2003). In general, refined grains are low in magnesium due to the food preparation processes that remove most of the magnesium. Magnesium content also becomes depleted during meal preparation, notably through the boiling of food (Vormann, 2003). The bioavailability of magnesium can be reduced by its interaction with other nutrients. For example, foods high in phytates can decrease magnesium absorption because they contain phosphate groups that bind to magnesium (Rimbach and Pallauf, 1999). However, in the average diet, the amount of phytate does not affect magnesium absorption (Bohn et al., 2004). Kim et al. (2007) evaluated the dietary intake for 3 days between the vegetarian (n=76) and omnivorous women (n-76) in Korea and found that the vegetarians consumed significantly larger amounts of magnesium than the omnivores.

The reference nutrient intake (UK RNI 300 mg/d for men, 270 mg/d for women) can be reached comfortably with daily intakes of magnesium-rich food sources. However, the average intake of magnesium in a Western diet typically does not exceed 260 mg for men and 200 mg for women (Vormann, 2003).

Table 1.1 Food sources of magnesium (USDA, 2017).

Food sources	Mg (mg) content per 100g weight
Pumpkin seeds	560
Brazil nuts	377
Cereals, ready to eat, (Kellogg's)	363
Chocolate	331
Almonds	268
Buckwheat flour, whole-groat	251
Pine nuts	250
Hazelnuts	162
Oat bran	156
Wheat flour, whole-grain	136
Soybeans	86
Spinach	82
Soy milk, 1 cup	61
Lima beans	56
Chickpeas	48
Brown rice	43
Okra	40
Potatoes	28

1.4.2 Magnesium Deficiency and Excess

Hypomagnesaemia, low magnesium levels in the blood, is unusual for those eating a healthy diet but can be present in humans when they consume a diet with low magnesium content for an extended period. The normal plasma magnesium concentration range is 0.75–1 mmol/L. Magnesium deficiency can develop even when plasma magnesium levels are within this range, because the body tends to extrude magnesium from bone and reduce magnesium excretion from the kidney in order to maintain the circulating concentration.

In laboratory animals, magnesium concentrations respond more rapidly and a decrease in plasma magnesium can be observed after a few days of eating a poor magnesium diet (Vormann, 2003).

There are other causes of magnesium deficiency, such as malabsorption, gastrointestinal and renal loss, liver or pancreatic disease, vomiting, diarrhoea and chronic alcoholism. Moreover, hypomagnesaemia may be induced by diabetes due to excess excretion associated with diuresis (Swaminathan, 2003). There are many symptoms related to hypomagnesaemia, including abnormal neuromuscular symptoms, gastrointestinal symptoms and personality changes (Mann and Truswell, 1998).

There is no reported hypermagnesaemia stemming from dietary sources alone because the kidneys excrete the excess magnesium in the urine. Therefore, only people with impaired kidney function are at risk of excess magnesium concentrations. High doses of magnesium supplements can cause diarrhoea, vomiting and nausea (Mann and Truswell, 1998).

1.4.3 Absorption and Excretion of Magnesium

The body typically maintains magnesium homeostasis by stabilizing magnesium absorption from the intestines and excreting excessive amounts through the kidneys. Magnesium is initially absorbed by the small intestine with most of the absorption taking place in the ileum and jejunum within the first six hours following consumption (Hardwick et al., 1991). 24-76% of dietary magnesium is absorbed in the gastrointestinal tract as an ion. There is a good correlation between magnesium absorption and the levels of magnesium in the body, as the highest absorption levels are related to the lowest magnesium status (Jahnen-Dechent and Ketteler, 2012, Vormann, 2003). This suggests that, as for other minerals, the expression and activity of transport proteins responds to circulating concentrations in order to maximise uptake from the diet. Magnesium is absorbed by a passive para-cellular pathway or an active transcellular pathway mediated by transient receptor potential melastatin-6 (TRPM6) (Stipanuk and Caudill, 2013) and transient receptor potential melastatin-7 (TRPM7) (Jahnen-Dechent and Ketteler, 2012).

The transient receptor potential (TRP) family is a superfamily of proteins that regulate ion passage through the membranes. The expression of TRP ion channels in cells is extensive (Touyz, 2008). TRP channels act as sensors to any cellular changes in ion concentrations, pH, pressure, temperature and osmolarity. As a response to such changes, the TRP channels can modulate several chemical and physical cellular functions, such as controlling ion homeostasis, growth, migration, differentiation and cell division. The TRP super-family includes eight sub-families, all of which resemble one another in terms of structure. One of these sub-families is melastatin (TRPM), which has eight members (Yee et al., 2014). TRPM1 Ca^{2+} entry channel; TRPM2 non selective channel activated by oxidative stress; TRPM3 found in brain and kidney Mn^{2+} and Ca^{2+} permeable (Touyz, 2008); TRPM4 and TRPM5 are impermeable to divalent cations; TRPM6 and TRPM7 primarily conduct Ca^{2+} and Mg^{2+}; TRPM8 non selective cationic pores (Yee et al., 2014).

TRPM7 is a cation channel that controls the Mg^{2+} flux in the cell, and it is expressed in vascular endothelial cells (Baldoli et al., 2013). The TRPM7 channel kinase consists of six transmembrane domains and α-kinase (Figure 1.7) (Yee et al., 2014). The TRPM7 channel is inhibited by intracellular free magnesium and magnesium-ATP (Paravicini et al., 2012, Schlingmann et al., 2007). An increase in Mg^{2+} concentration in the cell increases the phosphotransferase activity of the kinase, which decreases channel activity (Yogi et al., 2011, Touyz, 2008).

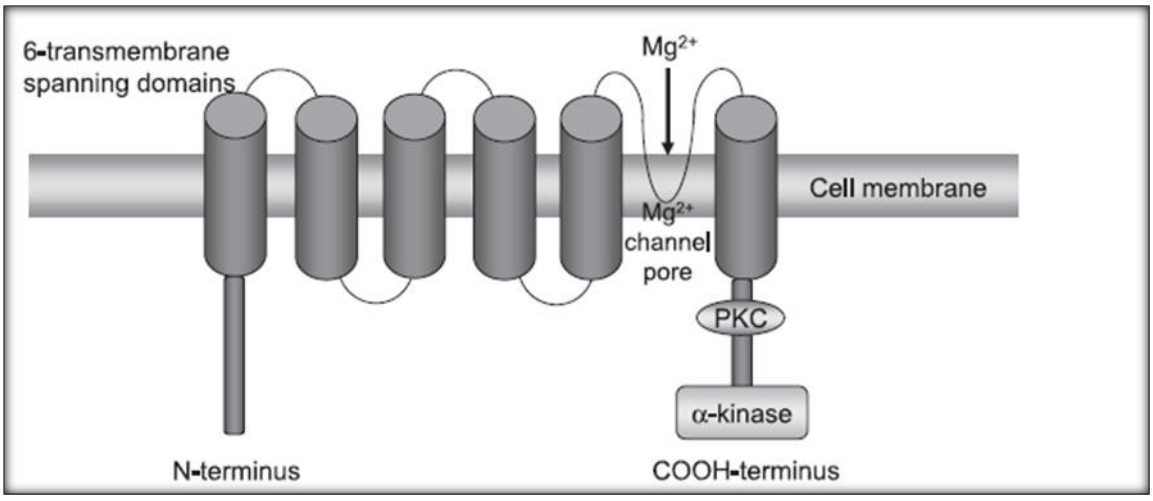


Figure 1.7 TRPM7 structure (Touyz, 2008).

The renal system also has an essential function in maintaining proper amounts of magnesium. It filters 75% of the plasma magnesium from the blood, reabsorbs 95% of that magnesium, and then the remaining is excreted in the urine. Generally, the control of magnesium homeostasis does not depend on any influencing hormones (Saris et al., 2000). However, as with gastrointestinal uptake, renal excretion of magnesium is responsive to magnesium status and is reduced when circulating concentrations are low (Coates et al., 2010).

1.4.4 Assessment of Magnesium Status and Treatment

Determining magnesium status in humans depends on an assessment of serum magnesium concentrations, which are usually stable. Magnesium concentrations in the serum generally do not exceed 0.3% of total body magnesium. However, this method may be inaccurate, as many people with magnesium deficiencies have normal serum magnesium levels (Noronha and Matuschak, 2002). Another method used to assess magnesium status includes the magnesium-loading test that measures the 24-hour urine excretion of magnesium. This test requires normal renal functioning and is not suitable for the elderly (Fawcett et al., 1999).

Clinical symptoms of magnesium deficiency are nausea, vomiting, loss of appetite and weakness. As magnesium deficiency worsens the symptoms are, numbness, tingling, muscle contractions and cramps, personality changes, abnormal heart rhythms, and coronary spasms develop (Saris et al., 2000). When magnesium deficiency symptoms do appear in patients, they should be treated with an oral supplement of 450 mg magnesium per day (Noronha and Matuschak, 2002). However, large doses can cause gastrointestinal side effects, and oral magnesium is poorly absorbed. Therefore, critically ill patients should be given magnesium by continuous intravenous administration (Swaminathan, 2003). There are a number of factors that increase the risk of magnesium deficiency including: alcoholism, Type 2 diabetes, renal disease and chronic gastrointestinal disease (Stipanuk and Caudill, 2013).

1.5 Magnesium, health and disease

Magnesium plays a role in maintaining health and deficiency can cause many disease states. Magnesium is linked to lung function, respiratory symptoms and airway reactivity. It can also be used to treat asthma (Saris et al., 2000). Magnesium is important for bone health and deficiency can be associated with osteoporosis (Swaminathan, 2003). Low magnesium levels are related to many neurological diseases, such as epilepsy, depression and migraines. Oral magnesium supplements can help relieve headaches and migraines (De Baaij et al., 2015). Low magnesium levels are also related to Alzheimer's disease. After evaluating the level of serum magnesium in 101 patients with Alzheimer's, it was discovered that the level of magnesium was lower than in the control group (Volpe, 2013).

1.5.1 Magnesium and Cardiovascular Disease

Low serum magnesium levels can result in cardiac arrhythmia due to the role of magnesium in potassium transportation through Na^+, K^+ ATPase pumps (Stipanuk and Caudill, 2013). The Health Professional Follow Up Study on 43,738 U.S. men found that there was an association between magnesium intake and hypertension, which can increase the risk of a stroke (Saris et al., 2000). Shechter (2010) mentioned that high magnesium levels can decrease the risk of myocardial infarction and coronary artery disease by inhibiting the thrombus formation, blood clots and platelet aggregation. Magnesium deficiency has been associated with congestive heart failure and ischemic heart disease (Champagne, 2008). Adamopoulos et al. (2009) conducted a follow-up study on 7,788 chronic heart failure patients for over 36 months. During this period, 327 patients died, with 74% of the deaths being due to cardiovascular diseases, 31% of these patients had low serum magnesium levels. This finding suggests that low serum magnesium levels may increase the risk of cardiovascular death.

1.5.2 Magnesium and cardiovascular disease risk factors

Low serum magnesium is related to many cardiovascular disease risk factors, such as type 2 diabetes, metabolic syndrome, arteriosclerosis and hypertension (Watanabe et al., 2011). Magnesium therapy inhibits myocardial apoptosis, cell adhesion and calcium build-up. Additional improvements can be observed in lipid metabolism, vascular tone and peripheral vascular resistance. Magnesium therapy is also associated with reduced free radical damage (Shechter, 2003).

Clues to an association between magnesium and CVD first came from studies examining links between water hardness and cardiovascular death (Ueshima, 2005, Chakraborti et al., 2002). A recent meta-analysis of nine articles including 77,821 cases with coronary heart disease Jiang et al. (2016) found an inverse relationship between magnesium levels in drinking water and coronary heart disease mortality, with RR 0.89 comparing highest with lowest concentrations. Similarly, a meta-analysis of 19 prospective cohorts, including over 53,200 subjects, found that the highest categories of magnesium intake and serum magnesium were associated with a lower risk of cardiovascular events (RR 0.85 for intake, 0.77 for serum concentration) (Qu et al., 2013). The results of a meta-analytic study of 34 trials containing 2028 participants indicated that magnesium supplementation for three months reduces systolic blood pressure (2 mmHg lower with 368 mg/day)(Zhang et al., 2016). Conversely, Joris et al. (2017) found that when 52 overweight patients were given 24 weeks of magnesium supplementation, either 350 mg/day or 3 doses of 117mg/day, there was no effect on cardiometabolic risk markers such as serum lipids, plasma glucose, plasma sVCAM-1 and sICAM-1. There was also no effect found on inflammation markers TNF, IL-8 and IL-6. These results were in comparison to a control group that received a placebo.

Randell et al. (2008) examined 1,318 subjects in Canada and determined that serum magnesium levels were negatively correlated with blood pressure, total cholesterol, fasting triglycerides (TG) and fasting glucose levels. By contrast, an increase in high-density lipoprotein (HDL) cholesterol levels was observed with high magnesium. A clinical study

observed that low serum magnesium levels in cardiovascular disease patients were associated with increased total cholesterol and that treating with magnesium was beneficial to protect from ischemic heart disease (Ueshima, 2005). Another important aspect of magnesium is the role it plays in reducing blood pressure. One study supporting the benefits of magnesium was conducted on 60 hypertensive patients, treated with 20 mmol/day (486 mg/ day) of magnesium oxide for eight weeks. This study found a notable decrease in blood pressure due to the reduction in sodium and calcium levels in endothelial cells (Houston, 2011). Furthermore, Hadjistavri et al. (2010) observed that hypertensive patients who took 600 mg of magnesium as a supplement had significantly decreased levels of total cholesterol, LDL cholesterol and TG and also increased HDL cholesterol levels after 12 weeks of magnesium treatment.

The Atherosclerosis Risk in Communities cohort study in the United States followed 15,792 women and men aged 45–64 years over a 4–7 year period. These researchers examined the association between magnesium and hypertension, diabetes and heart disease. Their results showed that low serum magnesium levels were associated with diabetes and hypertension, which, in turn, are related to ischemic strokes (Ohira et al., 2009). Essential hypertension patients treated with 600 mg magnesium oxide per day for eight weeks exhibited decreased blood pressure, total cholesterol, LDL and TG and increased HDL compared with the baseline (Annapurna et al., 2015). Hypertensive Japanese adults treated with magnesium (548 mg/day for males and 411 mg/day for females) showed decreased blood pressure and serum lipids compared with the controls (Champagne, 2008). Lecithin cholesterol acyl transferase (LCAT) activity requires magnesium, and this enzyme is necessary in increasing HDL levels (Rosanoff and Seelig, 2004).

Animal studies by King et al. (2009) showed that rabbits that fed on a high-cholesterol diet with magnesium supplementation for eight weeks exhibited increased HDL cholesterol levels and decreased TG and total cholesterol levels. Low magnesium leads to increased lipid deposition in the vascular wall, thus increasing the development of atherosclerotic plaques. Ravn et al. (2001) revealed that mice given magnesium sulphate in drinking

water for three months had decreased levels of TG and cholesterol when fed a high-cholesterol diet group. Additionally, macrophage staining of the aorta showed less plaque development in mice that drank water fortified with magnesium compared to those that drank tap water. These result suggests that magnesium is an anti-atherogenic element. An antecedent animal study indicated that low-magnesium diets for eight days decreased HDL cholesterol concentration in rat plasma, thereby resulting in increased plasma levels of TG and LDL (Rayssiguier et al., 1991).

Low serum magnesium levels are associated with insulin resistance as it is a cofactor for many of the enzymes that promote glucose homeostasis (Swaminathan, 2003). Additionally, the magnesium ion is required to activate insulin receptors that belong to the tyrosine kinase receptor family (De Baaij et al., 2015). Magnesium also promotes glucose translocation into the cells by influencing the activity of the insulin-regulated glucose transporter (GLUT4) protein (Volpe, 2013). Patients with type 2 diabetes show low serum magnesium levels (De Baaij et al., 2015). Treating type 2 diabetic patients with a 3 g magnesium supplement per day resulted in a slight but significant decline in plasma glucose (Ueshima, 2005). Treating 60 type 2 diabetic patients with 300 mg of magnesium chloride per day, for three months, resulted in enhanced insulin sensitivity when compared to a control group (Volpe, 2013).

Multiple mechanisms may explain the effect of magnesium on cardiovascular disease risk. These include the role of magnesium as a co-factor in many essential enzymes involved in lipid metabolism including, lipoprotein lipase and LCAT (Randell et al., 2008). Magnesium has also been suggested to regulate expression of peroxisome proliferator activated receptors which regulate fatty acid storage (King et al., 2009). Moreover, magnesium can inhibit the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme, an important enzyme in cholesterol synthesis (Kupetsky-Rincon and Uitto, 2012). Magnesium is also important in PGI2 production. PGI2 is an essential platelet inhibitor and vasodilator. Delta-6-desaturase enzyme requires magnesium as a co-factor in the process of producing gamma–linoleic acid, an important intermediate in prostaglandin synthesis

(Houston, 2011). Magnesium deficiency can also mediate changes in the cardiovascular system by influencing the movement of calcium, sodium and potassium through the sarcolemmal membrane, which affects both the specialised conducting system of the heart and the vascular smooth muscle tone. Magnesium can also affect the electrical activity of myocardial cells and alter myocardial contractility by controlling the intercellular calcium concentrations (Noronha and Matuschak, 2002).

1.5.3 Magnesium in cellular physiology

Magnesium is one of the most important intracellular cations. It plays a fundamental role as an essential controller of cell function. Within cells, magnesium ions are essential in regulating many biochemical reactions as a co-factor in more than 300 enzymes, which are required for protein, carbohydrate and lipid metabolism, nucleic acid synthesis, muscle contraction, neurotransmitter release and as a calcium antagonist (Fawcett et al., 1999). Magnesium is a required component for energy production, as the synthesis and use of adenosine triphosphate (ATP) require magnesium (Romani and Scarpa, 2000). In the cell, an important biological role of magnesium is for anion charge neutralisation. It binds to the phosphates of nucleotide di- and tri-phosphates. This process gives these nucleotides a negative charge which facilitates their binding to the enzymes that use them as substrates (Saris et al., 2000). Generally, magnesium can regulate enzyme activity through binding to the active sites and helping construct multi-enzyme complexes (Swaminathan, 2003). The activity of many of the enzymes that catalyse the transcription, translation and replication of DNA and RNA requires magnesium ions (Mg^{2+})(Chakraborti, 2002).

Magnesium regulates endothelial function by controlling cell growth. In addition, preventing oxidative stress and affect the production of NO and PGI2 that control vascular tone and blood pressure (Sontia and Touyz, 2007). Magnesium also influences cellular permeability and modulates the transport of potassium and Ca^{2+}. Additionally, magnesium ions can modulate the actions of hormones for example, insulin by serving as a second messenger (Chakraborti et al., 2002).

The total body stores of magnesium are between 20–24 g in an average 70 kg adult. Approximately 60% of the body's magnesium is in the bones, 35% in soft tissues such as the liver, heart, kidneys, brain and muscles (Shechter, 2010), and only 1% in the blood (James, 2010). An important aspect of magnesium homeostasis is the release from the bone surface to balance the extracellular magnesium concentration when serum magnesium concentrations are reduced. Therefore, bone mineral density is affected, making poor magnesium status one of the risk factors for osteoporosis (Vormann, 2003).

1.5.3.1 Magnesium–DNA interactions

DNA is composed of nucleotides, which are anions with more than one negative charge in their phosphate groups and require an opposite charge to stabilise the double helix. The double positive charge in Mg^{2+} generates a bridge among the phosphate groups, thereby creating the backbone of DNA. In addition, this binding decreases the density of the negative charge, protecting DNA from hydroxyl radicals (Anastassopoulou and Theophanides, 2002).

1.5.3.2 Magnesium and the fidelity of DNA synthesis

During cell division, the cell must pass on all the information that it's DNA carries to the new cells. Therefore, DNA polymerase translocates along the template and is copied as replication proceeds (Steitz, 1999). A number of metals can affect the accuracy of DNA replication (Zakour et al., 1981). To ensure the accuracy of the template during DNA synthesis, some steps require the DNA polymerase enzyme. These steps include reading the template strand, selecting the appropriate nucleoside triphosphate and inserting the correct nucleotide at the 3´ primer terminus. DNA polymerase is an enzyme–metal–substrate complex that requires an additional Mg^{2+} at two binding sites as a metal activator to catalyse the reaction (Steitz, 1999, Sirover and Loeb, 1976).

1.5.3.3 Magnesium and DNA repair processes

The DNA repair process corrects errors to ensure that DNA is replicated accurately. Errors can occur through either endogenous or exogenous physical or chemical processes. DNA damage in the form of mutations is typically generated through the replication of DNA, leading to many diseases (Adhikari et al., 2006).

Nucleotide excision repair (NER) is one of the most important processes that repairs damaged DNA. Damage is recognised by the ultraviolet–DNA damage binding protein. Then, the damaged area is cleaved by nuclease Xeroderma Pigmentosum Complementation Group G protein (XPG), and the helicase xeroderma pigmentosum group D (XPD) unwinds the dimer. Finally, gap filling is catalysed by DNA polymerase. All of these proteins require magnesium as a co-factor (Calsou and Salles, 1994). Base-excision repair is the quickest way to repair incorrect bases. It requires magnesium to activate the repair enzyme AP endonuclease and both the ligase and DNA polymerase enzymes, which repair the gap. In the mismatch repair (MMR) pathway, a protein called methyl-directed mismatch repair protein (MutL) recognises the mismatch. This protein requires ATPase activity, which needs Mg^{2+} to join the MutL-ATP connection (Hartwig, 2001).

1.5.4 The Interaction between Magnesium and Calcium

It has been established that the transportation of magnesium and calcium across the intestinal epithelial cells is shared, leading to competition between them for the carrier system. Thus, an increase in one affects absorption of the other (Dai *et al.,* 2007, Hardwick *et al.,* 1991). This interaction occurs in high concentrations from supplementation but not from the usual diet (Stipanuk and Caudill, 2013). The TRPM- 7 ion channel controls the balance between the two (Dai *et al.,* 2007).

1.5.4.1 Magnesium, Calcium and Vascular Endothelial Cell Function

Magnesium plays an important role in the preservation of intracellular Ca^{2+} concentrations, owing to competition between them to bind to the transport proteins (Swaminathan, 2003). Magnesium acts as a calcium channel blocker (Cunha *et al.,* 2012). NO synthase in an endothelial cell leads to the dilation of the blood vessel and NO is dependent on

calcium (Adamopoulos *et al.,* 2009). Indeed, the release of NO in the endothelial cell is both advantageous and disadvantageous. NO has a significant role in controlling blood vessel homeostasis, but an increase in NO is associated with oxidative stress through peroxynitrite formation. Hence, its production is dependent on calcium and an increase in intracellular calcium levels causes a corresponding increase in NO production (Dauphinee and Karsan, 2010, Saraswathi et al., 2004). The interaction between NO and oxygen-free radicals leads to the breakdown of NO, creating peroxynitrite, which causes endothelial cell dysfunction due to free radical cell injury. Thus, the balance between NO and the level of oxygen-free radicals is more critical than an elevation in NO levels, achieved by blocking the endothelial cell calcium channel (Kim *et al.,* 2002).

Calcium is a second messenger in the regulation of endothelial cell cytoskeletal function, thereby "opening" the intercellular junctions (Huang *et al.,* 1993). Calcium is important to barrier function in the endothelial cells through the regulation of cell-cell junctions (Brown and Davis, 2002). An increase in calcium levels leads to an improvement in endothelial cell permeability (De Bock et al., 2013, Moore et al., 2000). Cyclic adenosine monophosphate (cAMP) has a critical role to play in the regulation of the endothelial cell barrier. An increase in intracellular Ca^{2+} blocks the synthesis of cAMP by inhibiting the enzyme, adenylyl cyclase that is responsible for cAMP synthesis, which in turn, enhances barrier permeability and induces gap formation, resulting in a pro-inflammatory effect on the endothelial cells (Stevens *et al.,* 2000). The role of calcium as a second messenger is important in facilitating endothelial cell function because calcium promotes signalling transduction. Increasing the level of intracellular calcium activates the nuclear factor kappa B pathway and the production of adhesion molecules (Saraswathi *et al.,* 2004). A decrease in calcium levels is associated with a reduction in the pro-inflammatory response (Dai et al., 2007).

1.5.5 Low magnesium levels and vascular endothelial dysfunction

Vascular endothelial cells control thrombosis and thrombolysis, as well as the response to vasoconstrictors and vasodilators. Moreover, they act as a barrier to prevent molecules from exiting the blood (Sumpio et al., 2002). Endothelial dysfunction plays an essential role in vascular disease, and it is associated with the most common cardiovascular risk factors (Figure 1.8) (Ferrè et al., 2010, Shechter and Sherer, 2003). At a basic level, low extracellular magnesium has a negative impact on endothelial cell proliferation and inhibits cell migration (Banai et al., 1990). Migration is an important step for endothelial cells to achieve angiogenesis which is needed for tissue physiological requirement. To stimulate this migration, cells need to express phosphatases that demand a high concentration of extracellular magnesium (Leidi et al., 2010). The growth of endothelial cells is highly influenced by low magnesium concentrations. This is considered to be due to magnesium affecting DNA production and modulating specific genes that direct cell proliferation (De Baaij et al., 2015, Wolf et al., 2008a). Other studies have suggested that magnesium controls angiogenesis by decreasing the TRPM7, which controls endothelial proliferation and NO synthesis (Baldoli et al., 2013). Results *in vitro* provide evidence that culturing endothelial cells in a low magnesium concentrations inhibits proliferation after five days (Ferrè et al., 2007). This inhibition of proliferation is due to the impaired metabolism of carbohydrate and protein which is dependent on magnesium. All cell types need magnesium for the progression of their cell cycle (Maier et al., 2004a).

Fuentes et al. (2006) showed notable enhancements in endothelial function measured by a cardiovascular profiling instrument (model CR-2000), in patients with heart failure after treatment with 480 mg/d of magnesium for three months. Similarly, Barbagallo et al. (2010) treated elderly patients, with diabetes and hypertension, with 368 mg/d of magnesium for one month and found that oral magnesium significantly improved endothelial activity measured by flow-mediated vasodilation (FMD). Additionally, Shechter et al. (2000) examined 50 coronary artery disease patients given a placebo (n = 25) or 365 mg/d of oral magnesium (n = 25) for six months while continuing their usual diet.

This group measured endothelial function by FMD at the beginning and at the end of the treatment and found that oral magnesium enhanced the endothelial function of patients.

Several studies have observed that low magnesium in cultured endothelial cells contributes to inflammation and processes that may contribute to atherogenesis. These effects may be due to the activation of the transcription of the nuclear factor kappa NF-κB, which induces an overexpression of the inflammatory phenotype in endothelial cells (Ferrè et al., 2010, Rochelson et al., 2007). However, treating these cells with magnesium sulphate disrupts the action of NF-κB (Rochelson et al., 2007). Low magnesium in cultured endothelial cells up-regulates nicotinamide adenine dinucleotide phosphate-oxidase enzymes, which are a principal source of reactive oxygen species (ROS). The increase in ROS has been implicated in oxidation of LDL, that may promote atherogenesis and endothelial dysfunction by increasing inflammatory activity (Maier, 2012). Moreover, low magnesium affects the sensitivity of endothelial cells to the oxidant performance of hydrogen peroxide, which causes pro-oxidant effects (Figure 1.9) (Wolf et al., 2008b).

A study on 657 women who were not suffering from cancer, CVD, or diabetes indicated that low magnesium intake was associated with greater plasma concentrations of inflammatory cytokines and C-reactive protein (CRP), which are the common causes of endothelial dysfunction and atherosclerosis. The women that consumed magnesium-rich food had lower plasma concentrations of these inflammation markers (Song et al., 2007). A low-magnesium medium, in cultured endothelial cells, produce high amounts of IL-Iα protein in media, which affects cell proliferation by affecting the mRNA expression of many genes (Maier, 2012).

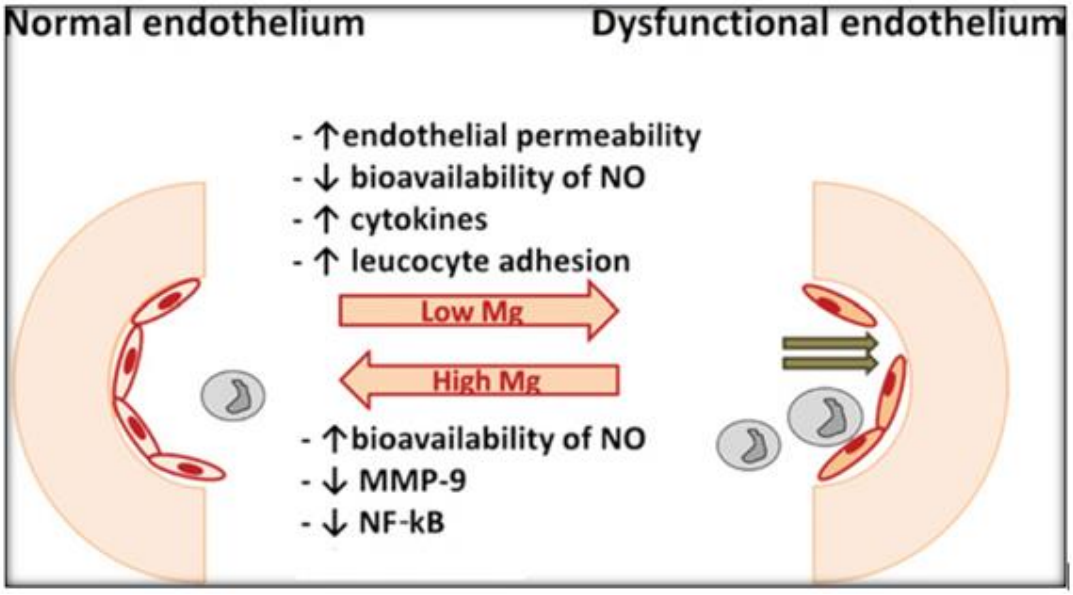


Figure 1.8 Effect of magnesium deficiency on endothelial function (Maier 2012).

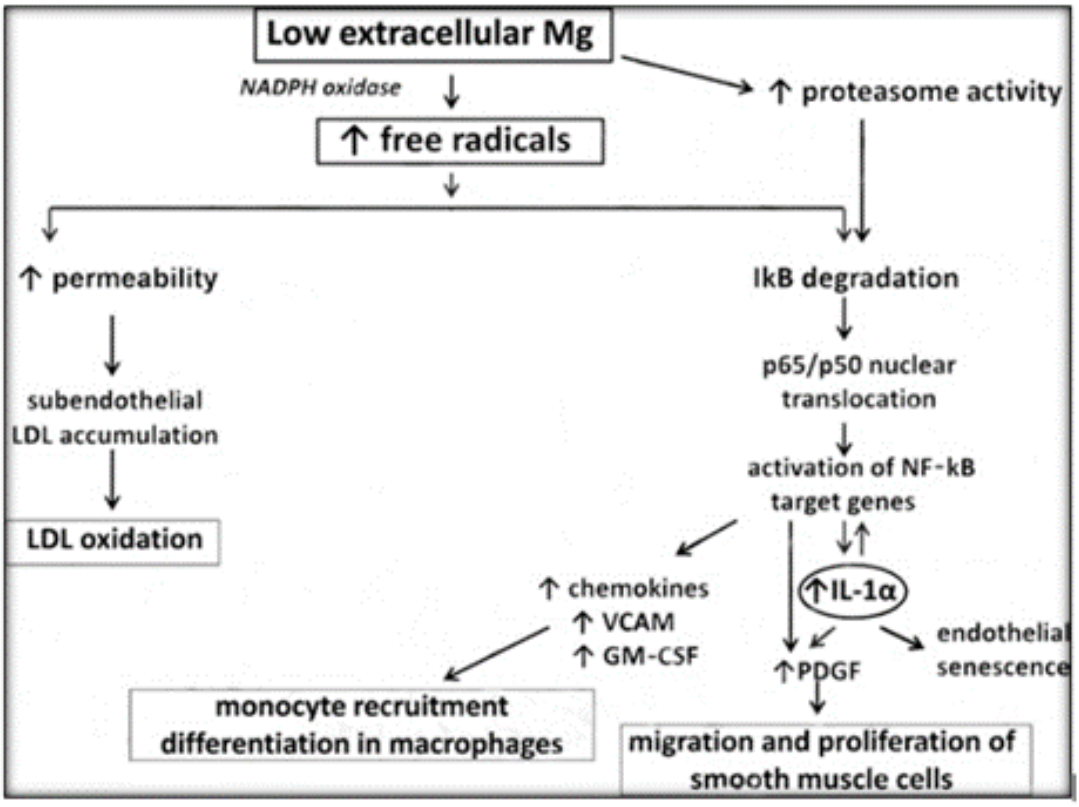


Figure 1.9 Effect of low magnesium on endothelial cells (Maier 2012).

1.5.6 Low Magnesium and inflammation

Low magnesium status is related to inflammation (Weglicki et al., 2010, King et al., 2009, Bernardini et al., 2005). The Harvard Nurses Study found a negative relationship between magnesium intake and the level of inflammatory response in plasma because of the anti-inflammatory effect of dietary magnesium (Galland, 2010). Similarly, the Nurse's Health Study showed that women who had a high magnesium intake had lower plasma inflammatory markers, such as CRP and E-selectin (Song et al., 2007). Furthermore, Sugimoto et al. (2012) collected blood samples from women before and after 6–16 hours of $MgSO_4$ therapy (6 mg/dl), and stimulated the samples with LPS. They found that magnesium therapy decreased the IL-6 and TNF response to LPS.

In addition, rat placentas collected 4 hours after injection of LPS with $MgSO_4$ showed reduced inflammation proteins IL-6, TNF-1 and CCL2 (MCP-1) in comparison to those from untreated rats (Dowling et al., 2012). Malpuech-Brugère et al. (2000) found that rats fed with a magnesium-deficient diet for four days had higher IL-6 in their plasma compared to controls. Kharitonova et al. (2015) also found that rats fed with a magnesium-deficient diet for 14 days had higher serum IL-6, CRP, VCAM-1, TNF-1 and endothelin and lower eNOS than the control rats. In an in *vitro* study, LPS stimulated human umbilical vein endothelial cells (HUVECs) cultured in low magnesium (0.1 mM) increased the production of VCAM-1, IL-1 and IL-6 in response to inflammation (Bernardini et al., 2005).

The mechanisms for the anti- inflammatory effect of magnesium are not clear (Sugimoto et al., 2012, King, 2009, Mazur et al., 2007, Malpuech-Brugère et al., 2000). One theory involves the role of magnesium as a calcium antagonist, and the role of magnesium in controlling cholesterol concentrations (King, 2009). Moreover, magnesium plays a part in inhibiting NF-κB activation (Sugimoto et al., 2012, Mazur et al., 2007).

1.5.7 Human umbilical vein endothelial cells (HUVECs) as a model of endothelial cell function

As previously discussed, vascular endothelial cells have an essential role in the regulation of blood flow and the maintenance of vessels during physiological haemostasis and

endothelial dysfunction is related to atherosclerosis and CVD. HUVECs are an easily obtained source of endothelial cells which are commonly used in *vitro* (Scott and Bicknell, 1993). As a result, much of our information about endothelial cells comes from research using HUVECs as a model (Bouïs et al., 2001). HUVECs have been used to study a wide range of biological and clinical processes including:

- cell proliferation and angiogenesis of wound –healing (Rhim et al., 1998).
- the migration and apoptosis of endothelial cells (Rhim et al., 1998).
- gene therapy targets for cancer and cardiovascular disease (Rhim et al., 1998).
- the involvement of inflammation in atherosclerosis (Onat et al., 2011).
- the role of diabetes in vascular inflammation (Onat et al., 2011).
- leukocyte adhesion and transmigration and to study the release of adhesion molecules and cytokine (Marin et al., 2001).

A limitation of using HUVECs as an in *vitro* model is that it may behave differently in *vivo*. This is because in *vivo*, there are interactions between endothelial and other surrounding cells and the different agents which may act upon them (Staton et al., 2009, Bouïs et al., 2001).

1.6 Aims, Hypothesis and Objectives

Magnesium is fundamental to normal cell function due to its role in DNA and protein synthesis. It is also an essential cofactor for numerous enzymes (De Baaij et al., 2015, Yang et al., 2004). Magnesium controls vascular tone by stimulating the response to vasodilators and vasoconstrictors. Moreover, it can protect vascular cells, due to its anti-inflammatory functions (Touyz, 2008). Clinical studies indicate that magnesium deficiency is implicated in increased risk of atherosclerosis and concomitant cardiovascular disease (Champagne, 2008, Wolf and Trapani, 2008, Sontia and Touyz, 2007, Maier, 2003). Low extracellular magnesium concentrations impact on endothelial cell proliferation; the production of nitric oxide; calcium release and inflammation, all of which are processes involved in the underlying pathology of many cardiovascular diseases (Maier et al., 2004b, Maier, 2003).

Based on the finding that endothelial dysfunction is an important factor associated with the development of atherosclerosis, and that magnesium plays a vital role in endothelial function, the main aims of this PhD study were to evaluate the effects of magnesium on the functioning of endothelial cells and to determine its potential anti-inflammatory role. This will be studied using the well characterized HUVECs culture model.

1.6.1 Hypothesis

Magnesium plays a critical role in maintaining normal endothelial cell function and low magnesium concentrations will promote biochemical changes associated with increased risk of cardiovascular disease.

1.6.2 Objectives

1- To determine the effect of magnesium concentration on HUVECs proliferation.

2- To identify the effects of magnesium concentration on global HUVECs gene expression.

3- To understand the anti-inflammatory role of magnesium on HUVECs.

2

Materials and Methods

2 Materials and Methods

2.1 Cell Culture

2.1.1 Overview

Primary Human Umbilical Vein Endothelial Cells (HUVECs) are extracted from the human umbilical cord vein and, as discussed in detail in section 1.5.7, represent an appropriate model in which to study the vascular biology and the endothelial functions including, proliferation, atherosclerosis and inflammation-related pathways (Marin et al., 2001, Chappey et al., 1995).

2.1.2 HUVECs Culture

HUVECs were purchased from Lonza (HUVEC-Umbil Vein, C2519A). The cells were cultured by transferring 1 ml (5×10^5 cells) of primary cells to T25 cm^2 culture flasks containing 5 ml of endothelial cell growth medium (EGM-2, Lonza), as shown in Table 2.1. Cells were incubated at 37°C (5% CO_2); the growth medium was changed 24 hours after seeding and the volume of the growth medium was increased to 10 ml and changed every other day until cells were grown to 80-90% confluence. The cells were used until passage 8. After that, the cells stop proliferating and start to die (Bouïs et al., 2001).

2.1.3 Sub-culturing HUVECs

HUVECs were sub-cultured when they were 80–90% confluent. The medium was aspirated and the cells were washed with 10 ml of warmed phosphate buffered saline (PBS, Sigma Aldrich, UK). After removing the PBS, 2 ml of 0.25% Trypsin-EDTA solution (Invitrogen) were added and incubated for 3-5 minutes to detach the cells from the surface of the flask. Then, 8 ml of EGM-2 was added, the solution was transferred to a sterile universal tube and centrifuged at 2500 rpm (Centrifuge-Eppendorf 5702R) for 5 minutes (at room temperature) to pellet the cells. The supernatant was aspirated, and then the cells were re-suspended in 10 ml of EGM-2. The required number of cells (50,000 cells per ml) was transferred to new 75 cm^2 flasks with 12 ml of EGM-2.

Table 2.1 Composition of Endothelial cell growth medium (EGM-2)*

Supplementation	EGM-2 (ml)	Final concentration* (% by volume)
EGM-2	500	
Fetal bovine serum (FBS)	10	2
Hydrocortisone	0.2	0.04
Human fibroblast growth factor (hFGF-B)	2	0.4
Vascular endothelial growth factor (VEGF)	0.5	0.1
Recombinant human long R3 insulin-like growth factor -1 (R3-IGF-1)	0.5	0.1
Ascorbic acid	0.5	0.1
Epidermal growth factor (hEGF)	0.5	0.1
Gentamicin amphotericin (GA-1000)	0.5	0.1
Heparin	0.5	0.1

*Absolute concentration is unknown as ingredients were supplied by the manufacturers.

2.1.4 THP-1 Culture

Human monocytic cells (THP-1), passage 2, were obtained from Dr M. Alcocer, School of Biosciences, University of Nottingham. THP-1 were cultured as suspensions in T25 cm^2 cell culture flasks in 10 ml RPMI-1640 medium (RPMI-1640, Sigma-Aldrich) supplemented with 10% FBS, 1% penicillin and 5×10^{-5} M 1,4-Dithiothreitol DTT, (Sigma Aldrich, UK). The THP-1 cells were incubated at 37°C (5% CO_2), and the volume of the growth medium increased by 5 ml twice a week until cell concentration reached 8×10^5 cell/ml.

2.1.5 Sub-culturing THP-1 Cells

THP-1 cells were sub-cultured when their concentration achieved 8×10^5 cell/ml. Then 1×10^5 cell/ml were taken from the cell suspension and seeded in T75 cm^2 cell culture flasks with 20 ml RPMI-1640 medium with 10% FBS, 1% penicillin and 5×10^{-5} M 1,4-Dithiothreitol (DTT).

2.1.6 Counting Cells

Cells were counted by adding 10 µl of the culture to a haemocytometer. Under the microscope, the cells in the four large corner areas were counted, and then the average was multiplied by 10^4 to get the number of cells per ml. To determine the volume of cell suspension needed for seeding density, the following formula was used:

Volume required (µl) = (Cell number needed / Total cell number) × Total volume of cells suspension (µl).

2.1.7 Cryopreservation

To store the cells for later experiments, seeded stock was frozen in liquid nitrogen at a low passage number. The cells were harvested (Section 2.1.3) and re-suspended in cold cryopreservation media (80% EGM-2, 10% FBS and 10% dimethyl sulfoxide [DMSO]; Sigma Aldrich). Then, 1 ml from the suspension containing 5×10^5 cells was pipetted into freezing vials, and the cells were frozen slowly at 1°C per minute using a cryo-freezing container at –80°C for at least 24 hours. The frozen cells were then stored in liquid nitrogen.

2.1.8 Preparation of Treatment Media

Different concentrations of magnesium sulphate ($MgSO_4$) were prepared in human endothelial serum and magnesium-free medium (SFM, Invitrogen USA). This medium required supplementation as shown in Table 2.2.

Table 2.2 Supplements in 100 ml magnesium-free medium

Supplementation	SFM	Final concentration
$MgSO_4$	1.203 mg 12.037 mg 60.185 mg	0.1 mM 1 mM 5 mM
Fetal bovine serum	10 ml	10%
Penicillin 100^X	1 ml	1%
Endothelial cell growth supplement (Sigma Aldrich, UK)	5 ml	5%

2.1.9 Experimental Protocol

HUVECs were cultured in EGM-2 media with 2% FBS and then incubated at 37°C (5% CO_2). When the cells reached 80% confluence, they were treated with SFM medium with varying concentrations of $MgSO_4$. Experiments were terminated at 24 or 72 hours after treatment, as described in the text in the results chapters of this thesis. The experiments used various sizes of dishes, cell numbers and medium volumes as shown in Table 2.3.

Table 2.3 the dishes size, the number of cells and volumes of medium for the experimental protocols

Dishes	Seeding density	EGM-2 medium	SFM medium
6-well	70,500	2 ml	2 ml
24-well	14,000	0.5 ml	0.5 ml
96 well	2,400	200 µl	200 µl

2.1.10 HUVECs Stimulation Assay

After plating out the cells as described in Section 2.1.9, the cells were treated with 0.1 mM, 1 mM or 5 mM $MgSO_4$ for 3 days. After that, the cells were either exposed to 0.5 µg/ml lipopolysaccharide (LPS, isolated from *Salmonella enterica* serotype *Enteritidis*, Sigma-Aldrich) for 4 hours or were untreated (Maier et al., 2004a).

2.2 Cell Viability Assays

2.2.1 Overview

As cell viability and proliferation is a fundamental part of cell biology, the following assays were performed to study the effect of $MgSO_4$ on endothelial cell proliferation.

2.2.2 Cell Proliferation

2.2.2.1 DNA Content Assay

Measuring the DNA content of a cell is one way to assess cell proliferation. The number of viable cells can be determined based on the fluorescent bisbenzimide dye solution, which binds to double-stranded DNA and gives a fluorescent emission that increases in the presence of DNA (Rage et al., 1990).

HUVECs were plated out in 96-well plates as described in Section 2.1.9, and then the medium was removed from the wells. The cells were washed with 100 µl of PBS, and 100

µl of sterile water were added to the wells. Next, the plates were incubated for one hour at 37°C (5% CO_2) and were then frozen at –20°C for 30 minutes. The plates were then defrosted and refrozen three times to lyse the cells and release the DNA.

Seven standard concentrations of calf thymus DNA (Sigma Aldrich, UK) were prepared which ranged from 0.312 to 20 µg/ml to create the DNA standard curve. The assay was performed in 96-well plates, with 100 µl of each standard. 10 µl of bisbenzimide dye solution (1 mg/ml stock, Sigma Aldrich, UK) were added to 1 ml of 10x TNE buffer (100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA) and 4 ml of sterile water in an aluminium-foil-wrapped bottle. 100 µl was added to each well, and the plates were then scanned using a fluorescent plate reader (CytoFluor™ 2350, Millipore Corp.) using 355-nm excitation and 465-nm emission wavelengths. A standard curve was produced using the calf thymus DNA standards to determine the DNA content of the cells in the wells.

2.2.2.2 Measurement of Total Protein Content – Lowry Method

The Lowry protein assay (Lowry et al. (1951) was used to estimate the amount of protein in the HUVECs. In this assay proteins are pre-treated with copper ions in an alkali solution. The amino acids in the sample reduce the acid in subsequently added Folin's reagent. The end product of this reaction is a blue colour and the amount of protein in the sample is estimated by reading its absorbance at 620 nm and comparing it against a standard curve of a selected standard protein solution.

HUVECs were cultured in 6-well plates as described in Section 2.1.9, and then the medium was removed from the wells. The cells were washed with 1 ml of PBS, and 0.5 ml of sterile water was added. The cells were scraped and transferred into Eppendorf tubes, and then the tubes were frozen at –20°C.

The Lowry reagents were prepared (Tables 2.4 and 2.5). Then, 50 µl of the standard (bovine serum albumin (BSA) 0.2-1.2 mg/ml) were added to the 96-well plates, followed by the loading of 25 µl of samples, the addition of 150 µl of 0.1 M sodium hydroxide (NaOH) to each standard well and the addition of 175 µl of 0.1 M NaOH to the sample wells. Reagent 1 (50 µl) was added to all of the wells. After 5 minutes, 50 µl of Reagent 2

was added. The absorbance was read after 20 minutes at 620 nm using a microplate reader (Bio-Rad Model 680XR).

Table 2.4 Composition of Lowry Reagent 1

Reagent	Volume required (ml)
2% sodium carbonate (Na_2Co_3) in 0.1 M of NaOH	5
1% Copper sulphate ($CuSO_4$)	0.5
2% potassium; sodium (KNa) tartrate	0.5

Table 2.5 Composition of Lowry Reagent 2

Reagent	Volume required (ml)
Folin–Ciocalteu phenol reagent	0.5
0.1 M NaOH	5

2.2.3 Cell Viability

2.2.3.1 MTT Assay

The MTT assay is a colorimetric method to determinate the viability of cells in culture. The yellow soluble tetrazolium salt, 3-(4,5-dimethylthiazol)-2,5 diphenyl tetrazolium bromide (MTT), is converted by metabolically active, viable cells into a purple-coloured insoluble formazan product (Twentyman and Luscombe, 1987).

HUVECs were cultured in 96-well plates as described in Section 2.1.9, and then the medium was removed from the wells. The cells were twice washed with 100 µl of PBS, and 100 µl of Hank's Balanced Salt Solution (HBSS; Sigma Aldrich, UK) without magnesium, 2% FBS and 20 µl MTT solution (5 mg/ml MTT in HBSS; Sigma Aldrich, UK) were added. Some wells were left empty (without cells) as blank. The plates were incubated for 4 hours at 37°C (5% CO_2). The HBSS was carefully removed, and 100 µl of DMSO added. The plates were covered and shaken gently for 15 minutes. Absorbance (590 nm) was

measured using a reference filter at 620 nm in a microplate reader (Bio-Rad Model 680XR). The results were quantified by comparing the absorbance values to 1 mM $MgSO_4$.

2.2.3.2 Trypan Blue Exclusion Assay

Trypan blue dye exclusion staining is a common method used to determine the number of viable cells in culture. The principle of this assay is that the living cell has intact cell membranes that prevent the trypan blue dye from entering, whereas the dead cells' loss of membrane selectivity allows the dye to bind to intracellular proteins giving the cell a blue colour (Maier et al., 2004b).

HUVECs were harvested as described in Section 2.1.3. Then, 10 µl of the cell suspension was mixed with 10 µl of 0.4% Trypan blue solution (Sigma Aldrich, UK) and pipetted onto the haemocytometer. The cells were examined under the microscope, and the number of viable and nonviable cells was counted. Live cells appear colourless and bright, and nonviable cells stain blue. The following equation was used to determine the viable cells:

% Viable cells = (Number of white cells / number of total cells) × 100.

2.3 Gene Expression Analysis

2.3.1 Overview

In this thesis quantitation of steady-state mRNA concentration was used as a measure of gene expression in HUVECs. This analysis was done by using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), which involves the synthesis and quantification of cDNA from mRNA extracted from the cells.

2.3.2 Effects of Various Concentrations of Magnesium on Human Endothelial Cells Gene Expression, Measured by Quantitative Real-Time PCR

HUVECs were plated out in 6-well plates as described in Section 2.1.9. At the end of the incubation period, the medium was removed from the wells, and 200 µl RNase-free PBS were added to each well. Next, the cells were scraped from the bottom of the wells and transferred into an autoclaved Eppendorf tube and straightaway put on ice to avoid RNA degradation. The samples were stored at –80°C for total RNA extraction.

2.3.3 Ribonucleic Acid (RNA) Extraction of HUVECs

RNA was extracted using a High Pure RNA Isolation Kit, following the manufacturer's protocol (High Pure; Roche, Mannheim, Germany, 2008). While still frozen, 400 µl of lysis/binding buffer was added to each sample. After vortexing for 15 secs the samples were transferred to the upper reservoir of the filter tube, which was inserted into one collection tube. The samples were centrifuged at 8,000 × g (Sigma, 1-16 SciQuip centrifuge) for 15 seconds (at room temperature for all centrifugations). After that, the filter tubes were removed from the collection tube. The flow-through liquid was discarded, and the contents of the filter tube and the used collection tube were combined again. To remove any contaminating DNA, 90 µl DNase I Incubation Buffer and 10 µl DNase I were added to each sample and incubated for 15 minutes at 15°C to 25°C. After incubation, 500 µl of Wash Buffer I were added to the upper reservoir of the filter tube assembly and centrifuged for 15 seconds at 8,000 × g. The flow-through liquid was discarded, and the contents of the filter tube and the used collection tube were combined again. Next, 500 µl of Wash Buffer II were added to the upper reservoir of the filter tube assembly and centrifuged for 15 seconds at 8,000 × g. The flow-through liquid was discarded, and, again, the filter tube and the used collection tube were combined. Next, 200 µl of Wash Buffer II were added to the upper reservoir of the filter tube assembly and centrifuged for 2 minutes at a maximum speed of 13,000 × g to ensure the removal of the remaining wash buffer. The collection tubes were discarded, and the filters were inserted into clean, sterile 1.5 ml Eppendorf tubes. Finally, the RNA was eluted in 50 µl of elution buffer and centrifuged for one minute at 8,000 × g. The purified RNA was present in the Eppendorf tubes (Figure 2.1) and then stored at –80°C for later analysis.

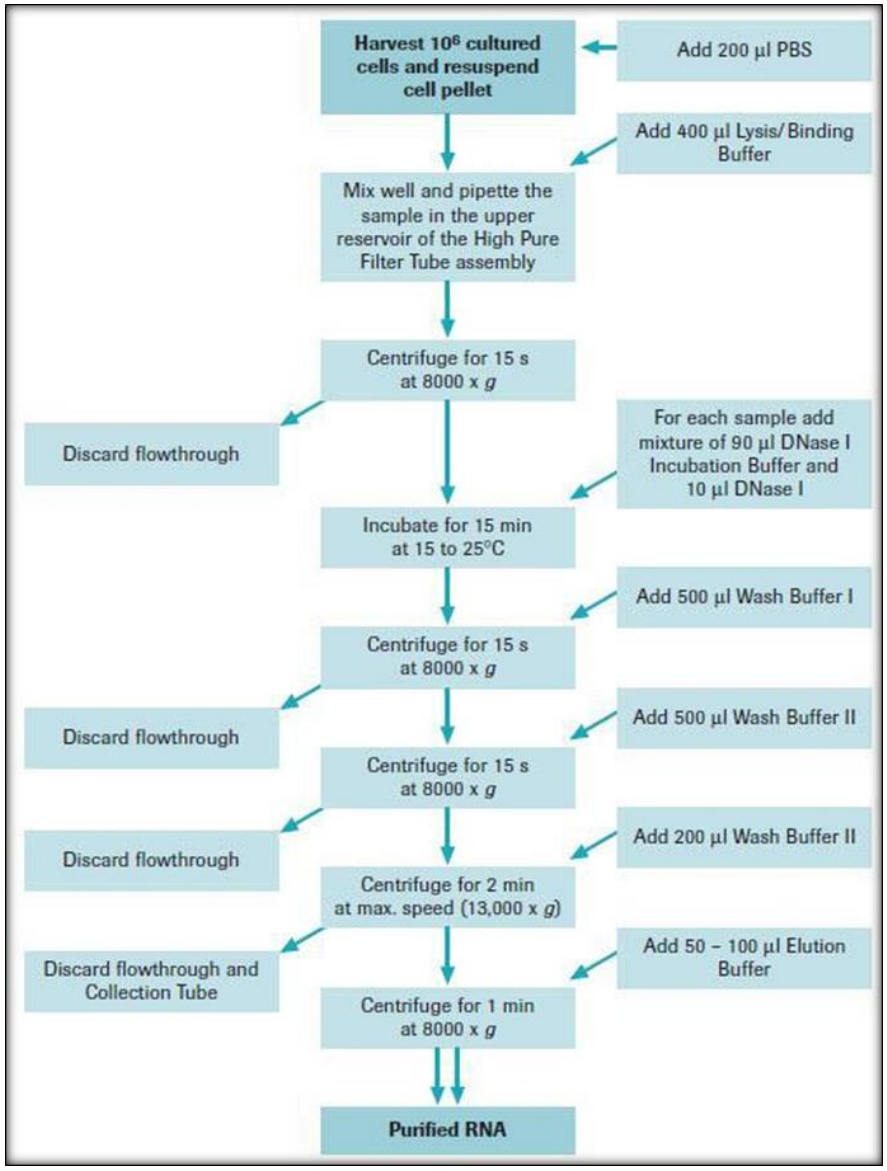


Figure 2.1 High Pure Isolation Kit Workflow (Roche 2008).

2.3.4 Determination of RNA Concentration

A Thermo Scientific Nano Drop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to quantify and determine RNA concentrations. It was blanked with elution buffer, and then 1.5 µl of the samples were measured. The ratio of the absorbance reading at 260 nm and 280 nm between 1.8 and 2.0 can be used to examine and assess the purity of RNA. Generally, the ratio of 260/280 is accepted at ~ 1.8 to 2.0 as pure RNA. If the ratio is lower than 1.8, this may reveal contamination with protein, phenol or other contaminants that absorb at or near 280 nm (Desjardins and Conklin,

2010, Pinto et al., 2009). All samples used had an RNA integrity ratio between 1.8 and 2.0. Sample concentrations were then adjusted to 50 ng/µl with RNase-free water.

2.3.5 RNA Integrity Check

RNA integrity was also tested by denaturing gel electrophoresis and running an aliquot of the RNA sample on an agarose gel (1% agarose gel was dissolved in 100 ml 1x TAE buffer) stained with ethidium bromide (EtBr). The gel was run at 100 V for 30 minutes. The bands can be seen under UV light, and Quantity-One software (Bio-Rad Laboratories, USA) was used to capture an image. The presence of discrete ribosomal RNA bands (18S and 28S) confirmed RNA integrity (Figure 2.2).

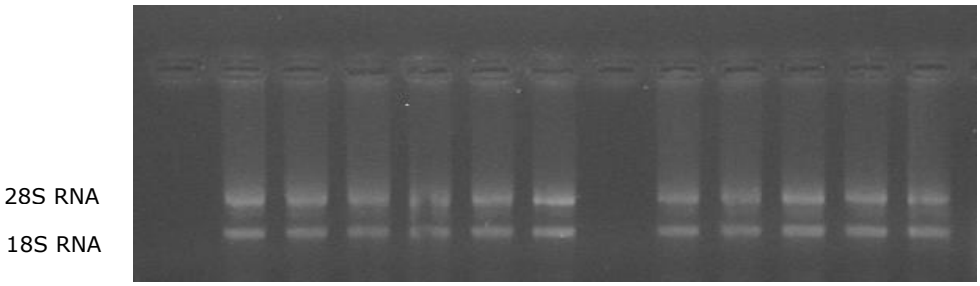


Figure 2.2 Example of HUVECs RNA samples on a 1% agarose gel
Ribosomal RNA (18S and 28S) bands were intact and not degraded.

2.3.6 Synthesis of cDNA

First strand DNA (cDNA) was synthesised with a ReverseAid RT Kit (Thermo Fisher Scientific 2013), following the manufacturer's instructions. This was done using reverse transcriptase, which is an enzyme that converts RNA into DNA. 10 µl of total RNA (50 ng/µl) was used with 1 µl random hexamer primer and 1 µl RNase-free water. This mixture was centrifuged briefly, incubated for 5 minutes at 65°C in a thermal block cycler and immediately placed on ice. The following components were then added: 4 µl of 5x reaction buffer, 1 µl of Ribolock RNase inhibitor (20 U/µl), 2 µl of 10 mM deoxynucleotide mix and 1 µl of ReverseAid Transcriptase RT (Table 2.6). This reaction was centrifuged briefly and incubated for 5 minutes at 25°C, followed by 60 minutes at 42°C. Finally, it was terminated by heating at 70°C for 5 minutes and placed directly on ice. A volume of 80 µl of RNase-free water was added to the 20 µl of cDNA to yield a final volume of 100 µl. Then, it was stored at –80°C until required for qRT-PCR.

Table 2.6 cDNA synthesis by reverse transcriptase

Materials	Volume for one reaction (µl)
Total RNA (50 ng/µl)	10
Random hexamer primers	1
RNase-free water	1
Transcriptor Reverse Transcriptase Buffer 5x	4
Ribolock RNase inhibitor 20 U/µl	1
10 mM Deoxynucleotide mix	2
ReverseAid Transcriptase RT	1
Total	20

2.3.7 Primer Design and mRNA quantification

Primer Express v3.0 (Applied Biosystems) was used to design primers for qRT-PCR. All sequences were taken from the National Centre for Biotechnology Information (NCBI). The design of the primers was confirmed using the Basic Local Alignment Search Tool (BLAST). Quantification of mRNA was performed using the standard curve method (Figure 2.3). All target genes were normalised to a cyclophilin housekeeping gene, which did not increase or decrease expression in response to any of the treatments in the experiments (See Appendix A,B & C). The primer sequences used are listed in Table 2.7.

A B

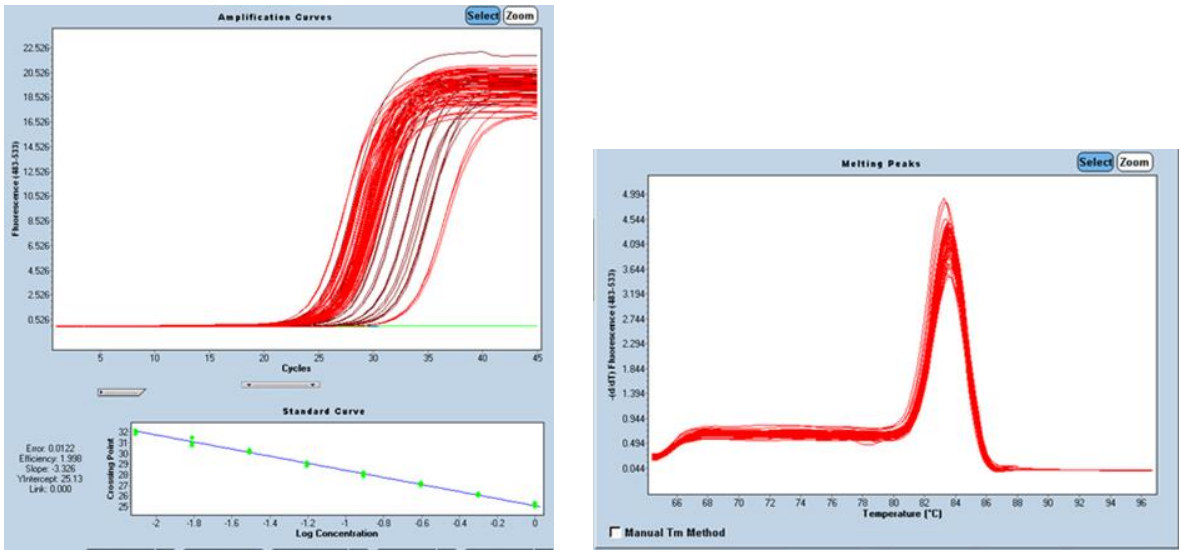


Figure 2.3 Examples of the Standard Curve Method for determining ICAM-1 gene. A: A dilution series of pooled cDNA is used to establish a standard curve in experimental samples. To confirm the reaction efficiency, the slope of the standard is a measure of reaction efficiency. The obtained slope is 3.32, and the efficiency is between 1.8 and 2. The brown lines indicate the standard curve. B: Melting-curve analysis is a simple way to check primer-dimer artefacts and to ensure reaction specificity. Only one melt curve peak was produced, indicating a single amplicon was generated in the PCR.

Table 2.7 Primer sequences used for qRT-PCR

Symbol	Gene name	Foreword primer	Reverse primer
TRPM7	Transient receptor potential cation channel, subfamily M, member 7	TTTTAATTTGGGCTTGCCTTATG	GGCAACTAATGCTTTAGCCATTG
eNOS	Nitric oxide synthase 3	GGTCTGCACAGGAAATGTTCAC	GATGGCCGAGCGAAGGTT
ET-1	Endothelin-1	CGTCCCTGATGGAAAGAGTGT	AACGTGCTCGGGAGTGTTG
IL-8	Interleukin 8	ACCGGAAGGAACCATCTCACT	ATCAGGAAGGCTGCCAAGAG
IRAK	Interleukin-1 receptor-associated kinase 1	CGCCCCTTTCCGTTTTG	GATCTTGAGCTCCTCCGAGAAG
TRAF6	TNF receptor-associated factor 6	TCAGTACTTTTGGTTGCCATGAA	AGCCTGGGCCAACATTCTC
IKB	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	GCTGAAGAAGGAGCGGCTACT	TCGTACTCCTCGTCTTTCATGGA
NF-κB	Nuclear translocation of nuclear factor kappaB	GGGATCTGCGCCGTTTCT	TCCCCATCATGTTCTTCTTAGTCA
TNF-Beta	Tumor necrosis factor Beta Lymphotoxin Alpha	TGTTGGCCTCACACCTTCAG	TGCTGTGGGCAAGATGCAT
IL-1B	Interleukin 1, beta	TCTGCCCAGTTCCCCAACT	TGGTCCCTCCCAGGAAGAC

TLR2	Homo sapiens Toll-like receptor 2	TCTCCAAGGAAGAATCCTCCAA	GAGCTGCCCTTGCAGATACC
TLR4	Homo sapiens Toll-like receptor 4	GGGTCCAACACTTGTTCAGTTAATAA	TGCATATCTAGTGCACCATGGAA
ICAM-1	Intercellular adhesion molecule	TCCCCCCGGTATGAGATTG	GCCTGCAGTGCCCATTATG
VCAM	Vascular cell adhesion molecule	GCAAGGTTCCTAGCGTGTAC	GGCTCAAGCATGTCATATTCAC
IL-6	Interleukin 6	CCGGGAACGAAAGAGAAGCT	GCGCTTGTGGAGAAGGAGTT
MCP-1	Homo sapiens chemokine (C-C motif) ligand 2 (CCL2)	CGCCTCCAGCATGAAAGTCT	GGAATGAAGGTGGCTGCTATG
PPI	Cyclophilin	GGAGATGGCACACAGGAGGAAA	CGTAGTGCTTCAGTTTGAAGTTCTCA

2.3.8 Measurement of Gene Expression by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RT-PCR was performed using the Roche LightCycler 480 (Roche, Burgess Hill, UK). It was programmed for an initial PCR activation step involving DNA polymerase enzymes via a heating step of 95ºC for 5 minutes. Forty-five cycles were performed that involved denaturation for 10 seconds at 95ºC then an annealing for 15 seconds at 60 ºC and elongation step for 15 seconds at 72ºC. A melting curve analysis was performed, which verified the product specificity (Figure 2.3). A 384-well white microplate was used with assays performed in triplicate. Firstly, all cDNA templates were diluted (1:8) in nuclease-free water. Then, 5 µl of diluted cDNA was mixed with the SYBR Green Master Mix (Roche, Table 2.8). Serial dilutions of a cDNA pool made from all samples were performed to

produce a standard curve for every gene and thus determine the efficiency and linearity of the PCR for each gene (Figure 2.3).

Table 2.8 SYBR Green Master Mix

Component	Volume (µl)
SYBR Master Mix Solution	7.5
Forward primer	0.45
Reverse primer	0.45
Water	1.6
Total volume	10

2.4 Microarray Analysis

2.4.1 Overview

The effect of magnesium on HUVECs gene expression was also studied using microarray analyses. The microarray analysis was performed at Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, School of Biosciences (we thank Dr. Marcos Castellanos for assistance and Affymetrix microarray and Partek analysis), using GeneChip® 3 ' IVT PLUS Reagent Kit (Affymetrix), GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix). An overview of the protocol is shown in Figure 2.4.

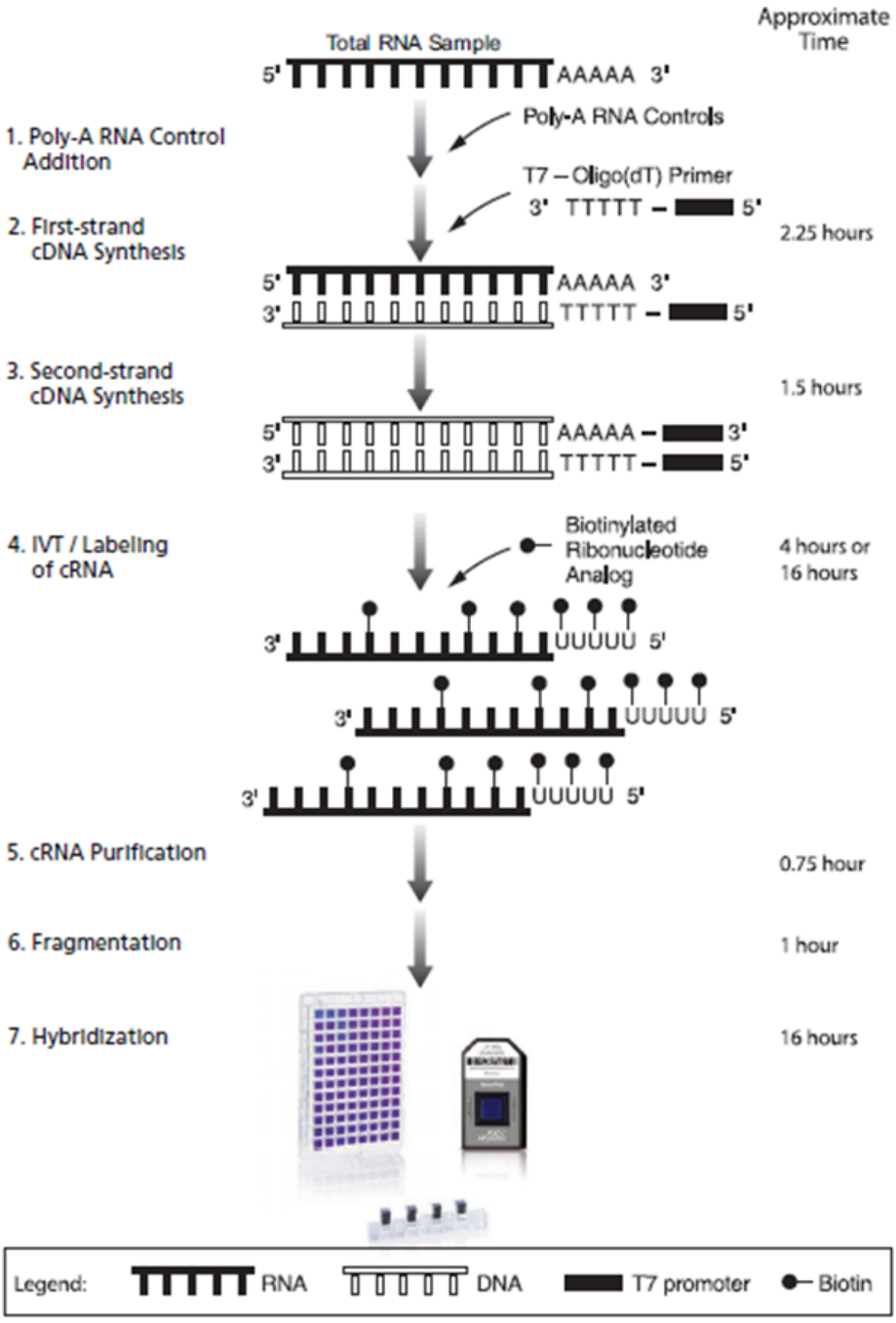


Figure 2.4 IVT Plus amplification and labelling process (Affymetrix)

2.4.2 Evaluation of RNA Quality and Integrity

RNA was checked to evaluate concentration using NanoDrop ND-1000 as described in Section 2.3.4. The integrity of the RNA was estimated using an Agilent RNA 6000 Nano Kit, 2100 Bioanalyser, and Agilent 2100 Expert Software, following the manufacturer's instructions (Agilent Technologies, Germany). First, the gel matrix was prepared by centrifuging 550 µl of RNA gel matrix through a spin filter 3660 rpm for 10 minutes. Next, gel-dye mix was prepared by adding 1 µl of RNA dye into 65 µl of filtered gel matrix, and then it was spun at 11000 rpm for 10 minutes. 9 µl of the gel-dye mix was loaded into the well Ⓖ in a new RNA nano chip (Figure 2.5) placed into the chip priming station. The chip priming station was closed and after 30 second the plunger was released. The chip priming station was then opened after pulling the plunger back to its original position, and 9 µl of the gel-dye mix was pipetted into the **G** wells.

After that, 5 µl of the RNA marker was placed in the ladder well and all of the sample wells. Then 1 µl of the samples were added to all sample wells and 1 µl of the RNA ladder to the ladder well. The chip was vortexed in an IKA vortexer for 1 minute at 2400 rpm. Finally, the chip was run in the 2100 Bioanalyser. Agilent 2100 Expert Software was used to analyse the electrophoresis run summary. The graph that resulted from the bioanalyser conveys similar information to running RNA on an agarose gel (Section 2.3.5), the X-axis denotes the time, which indicate fragment size. The amount of RNA was shown in the Y-axis. For each sample, the 2 peaks were present at 18s and 28s rRNA (Figure 2.6).



Figure 2.5. RNA nano chip

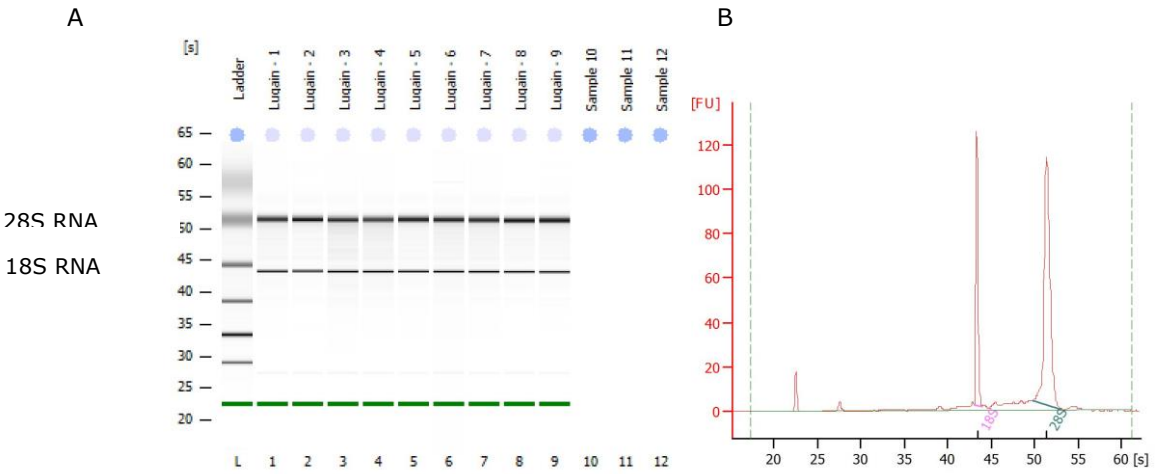


Figure 2.6 A- Agilent 2100 bioanalyser electrophoresis. Electropherogram traces of an Agilent RNA nano chip. B- The peaks refer to ribosomal 18S and 28S RNA

2.4.3 Preparation if Total RNA/poly-A RNA Control Mixture

Following the manufacturer's instructions GeneChip® 3 ' IVT PLUS Reagent Kit (Affymetrix). Poly-A RNA control was added to the RNA before the First Strand cDNA synthesis step. The mixture was done by adding 2 µl of diluted poly A-RNA control (4^{th} dilution) to 3 µl total RNA.

2.4.4 Synthesis of First Strand cDNA

Total RNA was reverse transcribed with T7 oligo (dT) primer. On ice, 5 µl of First Strand Master Mix (Table 2.9) was added to 5 µl of total RNA in a nuclease-free tube, and then mixed gently. The mixture was incubated for 2 hour at 42°C, then at 4°C for 2 minutes using the thermal cycler. After the incubation, the tubes were placed on ice for 2 minutes, and then the next step was done directly.

Table 2.9 First-Strand Master Mix

Component	Volume for One Reaction µl
3'First-Strand Buffer	4
3'First-Strand Enzyme	1
Total Volume	5

2.4.5 Synthesis of Second Strand cDNA

The First Strand cDNA was transformed to Second Strand cDNA by using DNA polymerase and RNase H. On ice, 20 µl of Second Strand Master Mix (Table 2.10) was added to 10 µl First Strand cDNA in a nuclease-free tube. In a thermal cycler, the samples were incubated for 1 hour at 16°C, then 10 minutes at 65°C, and after that for 2 minutes at 4°C. Then the samples were placed on ice for the next step.

Table 2.10 Second-strand Master Mix

Component	Volume for One Reaction µl
Nuclease-free water	13
3’ Second-Strand Buffer	5
3’ Second-Strand Enzyme	2
Total volume	20

2.4.6 Synthesis of Labelled cRNA

In a method called *in vitro* transcription (RT-IVT), cDNA Second Strand was used as a template to synthesize and amplify labelled cRNA by using T7 RNA polymerase. The procedure was started by adding 30 µl of IVT Master Mix (Table 2.11) to 30µl Second Strand cDNA samples. Then the samples were incubated in a thermal cycler at 40°C for 16 hours, then at 4°C for 2 minutes. The samples were frozen at -20°C for the next step.

Table 2.11 IVT Master Mix

Component	Volume for One Reaction µl
3’IVT Biotin Label	4
3’IVT Buffer	20
3’ IVT Enzyme	6
Total volume	30

2.4.7 Purification of Labelled cRNA

Labelled cRNA was purified for fragmentation by adding 100 µl of purification beads to a 60 µl cRNA sample, then mixed and transferred to U-bottom plate. Then it was incubated for 10 minutes to bind the cRNA in the sample to the purification beads. To catch the purification beads, the plate was set on a magnetic stand for 5 minutes. After that, the supernatant was discarded and 200 µl of wash solution containing 80% ethanol was added for 30 seconds. Then the wash solution was aspirated and the wash repeated three times. At the end of the washing step, the plate was air-dried for 5 minutes. Next, the plate was removed from the magnetic stand and 27 µl of pre- heated nuclease-free water was added and incubated for one minute. The plate was then placed on the magnetic stand for 5 minutes to catch the purification beads and the supernatant was transferred to a nuclease-free tube and stored at -20°C for the next step.

2.4.8 Fragment-Labelling of cRNA

The concentration of cRNA was measured using Nano Drop ND-1000 as described in Section 2.3.4, to determine the volume of cRNA needed in the fragment-labelled cRNA. On ice, the fragmentation reaction was mixed (Table 2.12). Then we used the thermal cycler to incubate the sample for 35 minutes at 95°C and for 2 minutes at 4°C; the sample was immediately placed on ice for the next step.

Table 2.12 Labelled cRNA Fragmentation Reaction

Component	Volume for One Reaction µl
Labelled cRNA (468.75 ng/µl)	32
3'Fragmentation Buffer	8
Total volume	40

2.4.9 Microarray Hybridization

Array analysis used the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, High Wycombe, Bucks, UK), which provides complete coverage of the human genome, plus 6,500 additional genes for total analysis of over 47,000 transcripts.

The array was pre-hybridized by filling it with 200 µl pre-hybridization mix, and then the array was incubated in an Affymetrix Hybridization oven at 45°C 60 rpm rotation for 10-30 minutes. Next the hybridization master mix was prepared by first heating the 20x hybridization control in the thermal cycler for 5 minutes at 65°C. After that, the rest of the components were added (Table 2.13). The hybridization cocktail then was prepared by adding 190. 6 µl of hybridization master mix was added to 29.4 µl fragmented and labelled cRNA, then incubated in the thermal cycler for 5 minutes at 99°C, then for 45°C for 5 minutes and centrifuge briefly.

The array was taken out of the hybridization oven and refilled with 200 µl of hybridization cocktail. Then the array was placed in the hybridization oven at 60 rpm for 4 to 16 hours at 45°C.

Table 2.13 Hybridization Master Mix

Component	Volume for one reaction µl
Control OligoB2 (3Nm)	3.7
20x Hybridization controls	11
2x Hybridization mix	110
DMSO	22
Nuclease-free water	43.9
Total volume	190.6

2.4.10 Microarray Wash, Stain and Scanning

After 4 hours the array was taken from the hybridization oven, the hybridization cocktail was extracted, and the array filled with wash buffer A. Affymetrix® GeneChip® Fluidics Station 450 was used to wash and stain the array. Finally, the array was scanned by an Affymetrix Scanner 3000 7G System.

2.4.11 Microarray Data Analysis

Affymetrix MAS 5.0 software was used to analyse the Affymetrix microarray. To identify differentially expressed genes between cells treated with different concentrations of magnesium, 0.1 mM and 5 mM treated cells were compared to the 1 mM group as a control. Corrected p-values of < 0.05 and Log2 fold-change greater than 1.5 were regarded as statistically significant. The data was then processed and subsequent analysis was done using Partek® Genomics Suite® software (Partek Inc., St. Louis, USA), which visualized the data.

2.5 Western Blot Analysis

2.5.1 Overview

Western blotting (or protein immunoblotting) is a technique used to measure protein expression in cell samples. The process consists of several steps. In this project Western blotting was performed essentially as described by Kurien and Scofield (2006).

2.5.2 Preparation of cell lysate

HUVECs were plated onto six-well plates as described in section 2.1.9. and the cells were grown to 90% confluence. The growth medium was replaced with 2ml of treatment media for each well and incubated for 24 hours and 72 hours (37°C, CO_2). At the end of the incubation period, the medium was removed from the wells, the cells were washed with cold PBS, and resuspended in 120 µl RIPA (radio-immunoprecipitation assay) buffer (Sigma Aldrich, UK) with (5 µl/ml) protease inhibitor cocktails (Calbiochem, USA) to prevent any protease enzyme action on the sample. The plate was incubated on ice for 30 minutes. Next, the cells were scraped from the bottom of the wells and the samples were transferred to an autoclaved Eppendorf and immediately placed on ice. The samples were centrifuged at 8000 xg for 10 minutes at 4°C to pellet the cell debris. Then the supernatant containing the soluble protein was transferred to an autoclaved 1.5 ml microcentrifuge tube. The samples were stored at -80°C to determine the protein concentration.

2.5.3 Protein Determination

Protein concentration was measured for each sample using a Lowry protein assay as described in section (2.2.5).

2.5.4 Electrophoresis

The samples were mixed with 6x sodium dodecyl sulfate (SDS)-mix Dithiothreitol (DTT) (5 volume of sample to 1 volume of SDS). 6x SDS was prepared as in (Table 2.14). The samples were boiled for 5 minutes at 90°C to denature the protein. 30 µg of the protein samples and 10 µl of protein marker ladder (Bio-Rad Precision Plus Protein Standard, Bio-Rad) were loaded onto a 4-10% mini Protean TGX precast polyacrylamide gradient gel (Bio-Rad), which was run in a tank filled with 1x running buffer (5 mM Tris, 50 mM glycine (Fisher Scientific) at 200V.

Table 2.14 6X SDS preparation

Chemical	Amount
Glycerol	6 ml
1M Tris/HCl	3.75 ml
SDS	1.2 g
DTT	0.462 g
Bromophenol Blue	Few grains

2.5.5 Transfer of proteins and staining (western blotting)

The proteins were transferred onto a nitrocellulose membrane (GE Healthcare) using the following method. The membrane was soaked in water for 10 minutes to activate. Then, the transfer cassettes contained the following layers: sponge, blotting paper, nitrocellulose membrane, gel, blotting paper and sponge (all equipment was pre-immersed in transfer buffer for at least 15 minutes). The transfer buffer contained 24 mM Tris, 192 mM glycine, 20% v/v methanol and distilled water. The transfer cassette was then placed in a transfer tank filled with transfer buffer for 2 hours at 200 mA with cooling. Following the transfer, the nitrocellulose membranes were washed with 1% Tween/1x Tris-buffered saline (TBS-T) washing buffer. To block non-specific binding sites, the nitrocellulose membranes were blocked for one hour with 2% ECL prime blocking reagent (GE Healthcare) in 1%

Tween/1x TBS (TBS-T) washing buffer. Next, the primary antibodies were diluted (Table 2.15) in 2% ECL prime blocking reagent in 1% Tween/1x TBS and incubated with the nitrocellulose membrane overnight at 4°C on a rocking table. Then, the nitrocellulose membranes were washed in TBS-T six times for 5 minutes each time. Then, the nitrocellulose membranes were incubated with horseradish peroxidase (HRP)-labelled anti-rabbit monoclonal secondary antibody at 1:8000 in 2% ECL prime blocking reagent in 1% Tween/1x TBS for one hour at room temperature. Next, the nitrocellulose membranes were washed in TBS-T six times for 5 minutes each time. After the detection was done (see Section 2.5.6), the nitrocellulose membranes were treated with restore Western blot stripping buffer (Thermo Scientific). α-tubulin primary antibodies (Sigma) were used as an internal control which stay stable with treatments, and they were incubated for one hour, at 1:10000. After washing, they were incubated with anti-mouse secondary antibody at a dilution of 1:40000. The proteins were detected using ECL Prime Western Blotting Detection Reagent kit.

Table 2.15 Antibody used for Western Blotting

Primary antibody	Supplier	Dilution	Description	Predicted band size kDa
Anti-TRPM7	Abcam	1/2000	Rabbit monoclonal	250
Anti-ICAM1	Abcam	1/2000	Rabbit polyclonal	57
Anti –eNOS3	Santa Cruz	1/5000	Rabbit polyclonal	150

2.5.6 Detection

The proteins were visualised by incubating the nitrocellulose membranes for 5 minutes in an enhanced chemiluminescence detection kit (ECL Prime Western Blotting Detection Reagent kit, GE Healthcare), and equal volumes were mixed from the two reagents. At the end of the incubation period, the membranes were dried to remove the excess reagent.

The membranes were then sealed in plastic cling film and placed in a film cassette. After that, the process was performed in a dark room. After placing the nitrocellulose membranes in a film cassette, a film (Hyperfilm, ECL Healthcare) was applied on top of the sheet protector, and the film was exposed for 1 to 30 minutes depending on the strength of the signal. The film was then developed by placing it in a developer tank (GBX Developer, Carestream Dental) for around one minute. It was then rinsed with water and put in a fixer tank (ILford Hypam Fixer, HARMAN Technology Limited, UK) for around one minute. Then, the film was air-dried. To obtain quantitative results from the Western blot film, Quantity One 4.2.1 Analysis Software (Bio-Rad) was used to detect the bands densitometry. The results were expressed in units relative to the internal control, α-tubulin.

2.6 Human Cytokine Array

2.6.1 Overview

The Human Cytokine Antibody Array Membrane kit (Abcam) was used to measure the protein expression of multiple cytokines simultaneously. This antibody pair-based assay is similar to ELISA, but uses a membrane rather than a microplate. Captured antibodies are distributed on the membrane. The procedure used for the array is shown in Figure 2.7.

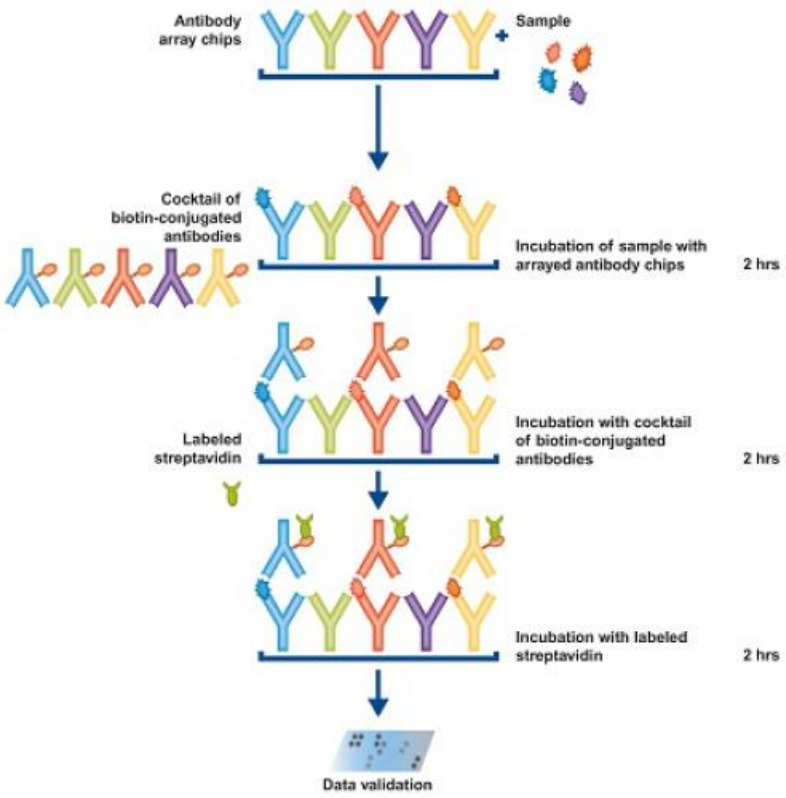


Figure 2.7 Process used with the Human Cytokine Antibody Array Membrane (Abcam).

2.6.2 Sample Preparation

HUVECs were plated onto six-well plates (section 2.1.9), and the cells were stimulated as explained in section 2.1.10. At the end of the stimulation time, the cells were lysed (section 2.5.2.) The amount of protein was determined using the Lowry protein assay (section 2.2.5).

2.6.3 Membrane Blocking and Incubation

The membrane was blocked according to the manufacturer's instructions (Human Cytokine Antibody Array Membrane kit; Abcam). First, the membrane was incubated with a 2 mL of 1x blocking buffer for 30 minutes. After the blocking buffer was aspirated from the membrane, the membrane was incubated overnight with 500 μg of protein in 1 ml of blocking buffer at 4°C on a rocking table. The membrane was then washed with wash buffer I for 45 min, after which it was washed with wash buffer II for 10 minutes. The

membrane was incubated for 1.5 hour with 1 ml of 1× Biotin-Conjugated Anti-Cytokines (2000× Biotin-Conjugated Anti-Cytokines vial in 2 ml of blocking buffer). It was then washed with wash buffer I for 15 min and wash buffer II for 10 minutes. The membrane was incubated for 2 hours with 2 ml of 1× HRP-Conjugated Streptavidin (1000× HRP-Conjugated Streptavidin vial in 1 ml of blocking buffer). At the end of the incubation, the membrane was washed with wash buffer I for 15 min and wash buffer II for 10 minutes.

2.6.4 Chemiluminescence Detection

Excess wash buffer was dried from the membrane by blotting the edges with tissue paper. Then 500 µl of equal volumes of detection buffer C and D mixed together and pipetted onto the membrane and incubated for 2 min. The detection was performed as described in section 2.5.6.

2.6.5 Obtaining Densitometry Data

To obtain densitometry data shown in Figure 2.8 and Table 2.16, Quantity One Analysis Software (Bio-Rad) was used to detect the spot signal densities. The results were expressed in units relative to the control spot provided with the biotin-conjugated IgG.

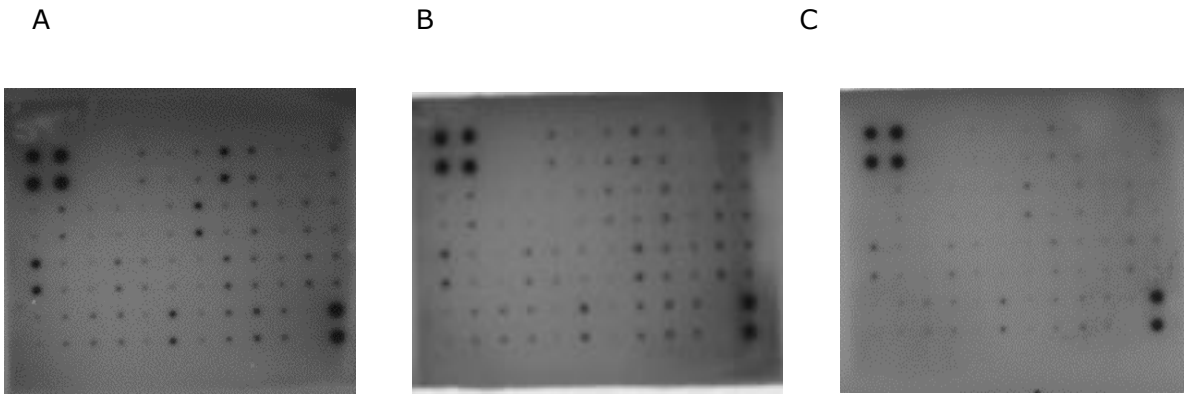


Figure 2.8 Representative of the Human Cytokine Antibody Array Membrane. A- 0.1 mM $MgSO_4$. B- 1 mM $MgSO_4$. C- 5 mM $MgSO_4$.

Table 2.16 Array Map

Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1 β
Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1 β
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 δ	RANTES	SCF	SDF-1	TARC	TGF-β1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1 δ	RANTES	SCF	SDF-1	TARC	TGF-β1
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoietin	VEGF	PDGF BB	Leptin	Neg	Pos

2.7 NFκB Pathway Array

2.7.1 Overview

The Human NFκB Pathway Array kit (R&D Systems, Ltd.) was used to quantify the expression of the proteins involved in the NFκB pathway. The array uses capture antibodies spotted on a nitrocellulose membrane.

2.7.2 Sample Preparation

HUVECs were plated onto six-well plates (section 2.1.9), and the cells were stimulated (section 2.1.10.) At the end of the stimulation time, the cells were washed with cold PBS, and then 120 µl lysis Buffer 6 (Abcam) was added with 5 µl/ml of protease inhibitor cocktail (Calbiochem, USA) to prevent any protease enzyme action on the sample. The plate was incubated on ice for 30 minutes. Next, the cells were scraped from the bottom of the wells, and the samples were transferred to an autoclaved Eppendorf and immediately placed on ice. The samples were centrifuged at 14000 ×g for 4 minutes at 4°C to pellet the cell debris. The supernatant containing the soluble protein was transferred to an autoclaved 1.5 ml microcentrifuge tube. The amount of protein was determined using the Lowry protein assay (section 2.2.5).

2.7.3 Membrane Blocking and Incubation

The membrane was blocked according to the manufacturer's instructions (Human NFκB Pathway Array kit; R&D Systems, Ltd.). The membrane was blocked for one hour with

array buffer 3/6 (equal volumes of array buffer 3 and array buffer 6 mixed together). Then the membrane was incubated overnight with 100 μg protein in 1.5 ml of array buffer 1 at 4°C on a rocking table. After incubation, the membrane was washed with 1× wash buffer (40 ml of 25× wash buffer in 960 ml of distilled water) for 10 minutes; there was a total of three washes. Then the membrane was incubated for one hour with 1.5 ml of diluted detection antibody cocktail (15 µl of detection antibody cocktail in 1.5 ml of array buffer 3/6). Next, the membrane was washed as described in the previous step. The membrane was then incubated for 30 minutes with 2 ml of 1:2000 diluted Streptavidin HRP in array buffer 3/6. Finally, the membrane was washed as explained in a previous step.

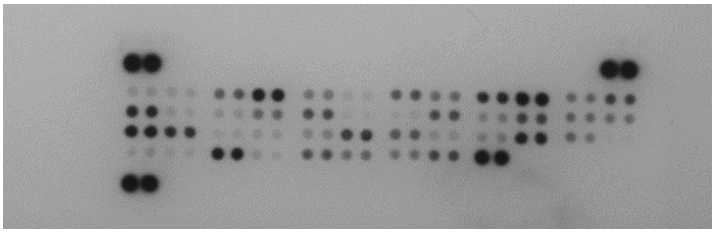
2.7.4 Detection

Excess wash buffer was dried from the membrane by blotting the edges with tissue paper. Then 1 ml of equal volumes of Chemi Reagent 1 and Chemi Reagent 2 mixed together was pipetted onto the membrane and incubated for one minute. The detection was performed as described in section 2.5.6.

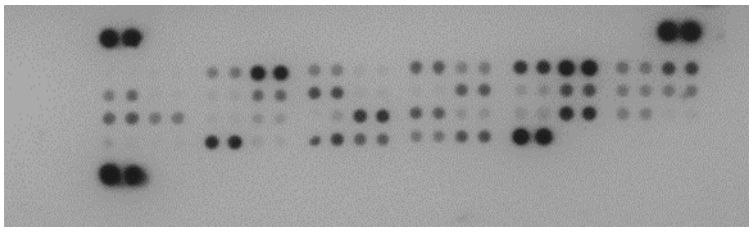
2.7.5 Data Analysis

To obtain densitometry data shown in Figure 2.9 and Table 2.17, Quantity One Analysis Software (Bio-Rad) was used to detect the spot signal densities.

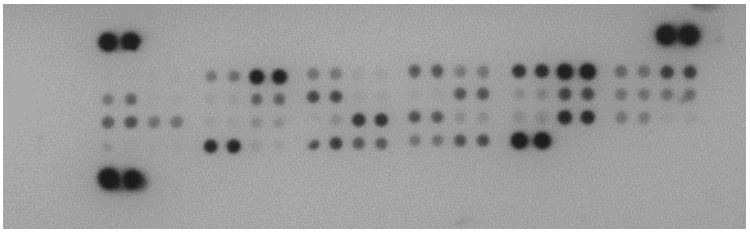
A



B



C



D

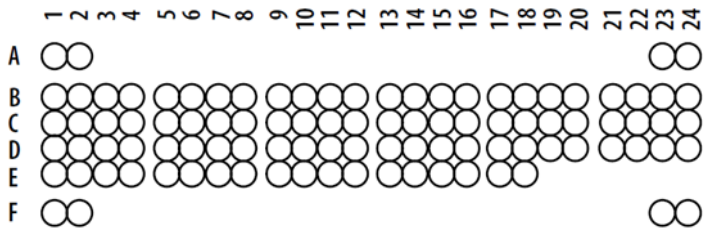


Figure 2.9 Representative of the Human NFκB Pathway Array Membrane. A- 0.1 mM $MgSO_4$. B- 1 mM $MgSO_4$. C- 5 mM $MgSO_4$. D- Human NFκB Pathway Array coordinates.

Table 2.17 Human NFκB Pathway Array coordinates

Coordinate	Target/Control	Coordinate	Target/Control
A1, A2, A23, A24	Reference Spots	C23, C24	MYD88
B1, B2	ASC	D1, D2	NFκB1
B3, B4	BCL10	D3, D4	NFκB2
B5, B6	CARD6	D5, D6	NGF R/TNFRSF16
B7, B8	CD40/TNFRSF5	D7, D8	p53
B9, B10	cIAP1/BIRC2	D9, D10	p53 (pS46)
B11, B12	cIAP2/BIRC3	D11, D12	RelA/p65
B13, B14	FADD/MORT1	D13, D14	RelA/p65 (pS529)
B15, B16	Fas/TNFRSF6/CD95	D15, D16	c-Rel
B17, B18	IκBα	D17, D18	SHARPIN
B19, B20	IκBε	D19, D20	SOCS6
B21, B22	IKK1/IKKα/CHUK	D21, D22	STAT1p91
B23, B24	IKK2/IKKβ	D23, D24	STAT1 (pY701)
C1, C2	IKKγ/NEMO	E1, E2	STAT2
C3, C4	IL-1 RI	E3, E4	STAT2 (pY689)
C5, C6	IL-17 RA	E5, E6	STING/TMEM173
C7, C8	IL-18 Rα	E7, E8	TLR2
C9, C10	IRAK1	E9, E10	TNF RI/TNFRSF1A
C11, C12	IRF5	E11, E12	TNF RII/TNFRSF1B
C13, C14	IRF8	E13, E14	TRAF2
C15, C16	JNK1/2	E15, E16	TRAIL R1/DR4
C17, C18	JNK2	E17, E18	TRAIL R2/DR5
C19, C20	LTBR/TNFRSF3	F1, F2	Reference Spots
C21, C22	Metadherin/AEG-1	F23, F24	Negative Control

2.8 TLR-2 and TLR-4 Blocking Assays

2.8.1 Overview

TLR-2 and TLR-4 blocking assays were performed to study the role of the TLR in LPS signalling to produce inflammatory cytokines. The assay was also used to assess the effect of varying magnesium concentrations on the response of HUVECs to LPS. This method was adapted from the report of (Satta et al., 2011) .

2.8.2 Blocking Assay

HUVECs were plated onto six-well plates (section 2.1.9), and the cells were treated with 0.1 mM, 1 mM or 5 mM $MgSO_4$ for 3 day. The medium was then removed from the wells, and the cells were washed with PBS. Next, the cells were blocked for 30 minutes using

10µg/ mL neutralising antibodies (Anti-TLR-2 or Anti-TLR-4; InvivoGen, USA), Anti-TLR2 and Anti-TLR4 together; some cells were not blocked and were used as a control. The cells were then stimulated with LPS as explained in section 2.1.10. Culture media were collected for ELISA, and cells were collected to measure the amount of cellular protein. In a separate experiment, the cells were collected for gene expression analysis.

2.9 Enzyme-linked Immunosorbent Assay (ELISA)

2.9.1 Overview

The Human CXCL8/IL-8 Quantikine Immunoassay kit and the Human VCAM-1 DuoSet kit (R&D Systems, Ltd.) is a quantitative sandwich enzyme immunoassay microplate-based technique. These kits were used to measure IL-8 and VACM-1 in cell culture supernatants. For both assays, a microplate was precoated with analyte of interest monoclonal antibody, which bonded with a target analyte in the samples. Detection of the bound to the primary antibody was assessed through the addition of a second horseradish peroxidase antibody and the addition of reagent to produce a coloured reactant that can be determined by spectrophotometry (Figure 2.10).

2.9.2 Sample Preparation

HUVECs were plated onto six-well plates (section 2.1.9), and the cells were blocked for 30 minutes using neutralizing antibodies (Anti-TLR-2 or Anti-TLR-4; InvivoGen, USA) as described in section 2.8.2. The cells were then stimulated with LPS (section 2.1.10). At the end of the stimulation time, the culture media were collected, and cells were lysed (section 2.5.2). The amount of protein was determined using the Lowry protein assay (section

2.2.5).

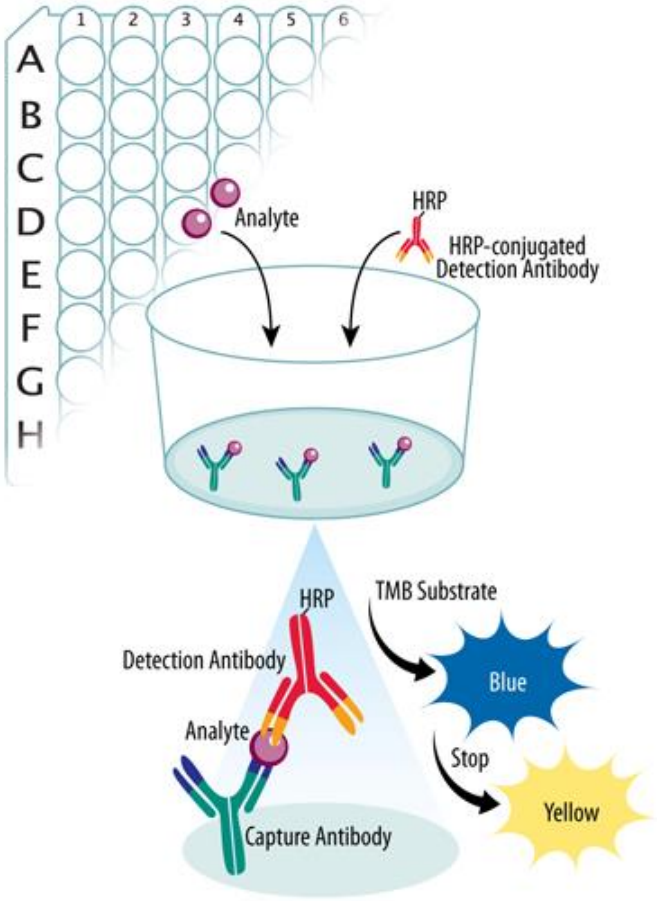


Figure 2.10 Process used for ELISA (R&D Systems).

2.9.3 Interleukin 8 ELISA Assay

The assay was conducted by following the manufacturer's protocol (Quantikine ELISA Human CXCL8/IL-8 Immunoassay, R&D Systems, Ltd). The assay began by adding 100 µl of Assay Diluent RD1-85 to each well. Then 50 µl of the standard or sample was added to each well and incubated for two hours at room temperature. At the end of the incubation period, the wells were aspirated and washed with 400 µl of wash buffer for a total of four washes. After the last washing step, any remaining wash buffer was aspirated. The plate was then blotted using clean paper towels. Next, 100 µl of human IL-8 conjugate was added to each well and incubated for one hour at room temperature. We then performed the washing step that was previously described. After the washing, 200 µl of substrate solution was added to each well and incubated for 30 minutes at room temperature. The plate was covered with foil to protect it from light. During the final step, 50 µl of Stop

Solution was added to each well, and the colour changed from blue to yellow. The optical density was measured using a microplate reader (Bio-Rad Model 680XR) set to 450 nm. The concentration of IL-8 in the samples was calculated from a standard curve of the human IL-8 standard.

2.9.4 VCAM-1 ELISA Assay

Levels of VCAM-1 were measured by following the manufacturer's instructions (Human VCAM-1/CD106 DuoSet ELISA, R&D Systems, Ltd). Each well of a 96 well microplate was coated with 100 µl of the diluted capture antibody (2 µg/mL in PBS) and allowed to remain at room temperature overnight. The plate was washed three times with 400 µl of wash buffer and blotted using clean paper towels. Next, 300 µl of Reagent Diluent was added to each well to block the plate. After incubation for one hour, the washing step was repeated. Then, 100 µl of either standards or samples was added to each well, and the plate was incubated for two hours at room temperature. After three washes with the wash buffer, 100 µl of diluted Detection Antibody (200 ng/mL in Reagent Diluent) was added to each well, and the plate was incubated for an additional two hours at room temperature. The washing step was performed again, and 100 µl of diluted Streptavidin-HRP (1:200 in Reagent Diluent) was added before incubation for 20 minutes in the dark at room temperature. After washing the plate, 100 µl of Substrate Solution was added and the plate was again incubated in the dark for 20 minutes. Then, 50 µl of Stop Solution was added, and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Model 680 XR). The concentration of VCAM-1 in the samples was calculated using the standard curve of human VCAM-1.

2.10 Cell Adhesion Assay

2.10.1 Overview

The Vybrant™ Cell Adhesion Assay kit (V13181, Molecular Probes, ThermoFisher Scientific) was used to evaluate the adherence of monocytes to HUVECs. The adhesion of monocytes to vascular endothelial cells in response to inflammatory cytokines is a key step in atherosclerosis (Akeson and Woods, 1993). The procedure used fluorescent dye to label

the monocyte cells, which were then added to culture HUVECs. The fluorescence of the adhering cells was measured using a fluorescence microplate reader (Figure 2.11).

2.10.2 Cell Culture

HUVECs were cultured onto 24-well plates as described in section 2.1.9, and the cells were treated with 0.1 mM, 1 mM or 5 mM $MgSO_4$ for 3 days. Next, the cells were stimulated as explained in section 2.1.10. At the end of the stimulation time, the cells were washed with warmed RPMI medium to be ready for the assay.

THP-1 cells were cultured in T75 cm^2 cell culture flasks as mentioned in section 2.1.5 until the cell concentration reached 8×10^5 cell/ml.

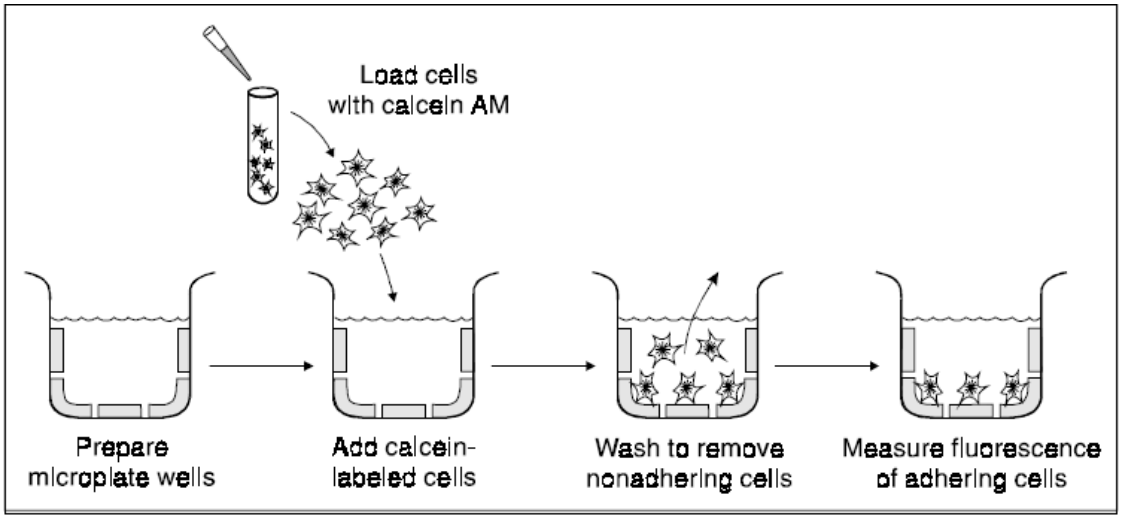


Figure 2.11 Process for the Vybrant Cell Adhesion Assay (ThermoFisher Scientific).

2.10.3 Calcein AM Labelling of Cells

THP-1 cells were labelled according to the manufacturer's protocol (Vybrant™ Cell Adhesion Assay kit; Molecular Probes, ThermoFisher Scientific). The cells were washed with PBS twice, then resuspended in RPMI medium without serum at 5×10^6 cells/ml. Then 5 µl of 1 mM calcein acetoxmethyl ester (calcein AM) was added to 1 ml of the cell suspension to produce 5 µM concentration, which was incubated for 30 minutes at 37°C (5% CO_2). The cells were then washed twice with warmed RPMI medium to stop the labelling, and resuspended at 5×10^6 cells/ml in the RPMI medium.

2.10.4 Monocyte Adhesion Assay

Following the manufacturer's protocol (Vybrant™ Cell Adhesion Assay kit; Molecular Probes), 500 µl of calcein-labelled THP-1 cells were added to each well containing HUVECs, and then incubated for one hour at 37°C (5% CO_2). The cells were then washed with warmed RPMI medium for a total of four washes to remove any nonadherent calcein-labelled cells. Then 1 ml of PBS was added to each well, and the fluorescence was measured using a fluorescence microplate reader (CytoFluor™ 2350, Millipore Corp.) using 494-nm excitation and 517-nm emission wavelengths. The percentage of cell adherent was calculated by dividing the fluorescence values of each well by the controls (total fluorescence of the labelled cell before adhesion) and multiplying by 100%.

2.11 Nitric oxide (NO) production measurement

2.11.1 Overview

NO was analysed using the Sievers Nitric Oxide Analyzer (NOA 280i). This method relies on the reaction of nitric oxide with ozone (O_3) to form nitrite (NO_2^-). Energy released as light is correlated with the initial concentration of NO.

2.11.2 Sample preparation

HUVECs were plated onto six-well plates (Section 2.1.9) and stimulated (Section 2.1.10). At the end of the stimulation period, culture media were collected and then stored at −20°C for NO analysis. The cells were lysed (Section 2.5.2) and analysed for proteins using the Lowry protein assay (Section 2.2.5)

2.11.3 Reagent preparation

2.11.3.1 Preparation of nitrite standards

According to the Sievers Nitric Oxide Analyzer (NOA 280i) manufacturer's instructions, a stock solution of 100 mM nitrite (Sigma-Aldrich, UK) was used to make six serial dilutions with concentrations from 1 nM to 500 nM.

2.11.3.2 Nitrite reducing agent preparation

To make the reducing agent which converts nitrite to nitric oxide, 50 mg sodium iodine was added to 0.5 mL pure water and 4.5 mL acetic acid. Then, 5 mL of the reducing agent was added to the purge vessel (Figure 2.12).

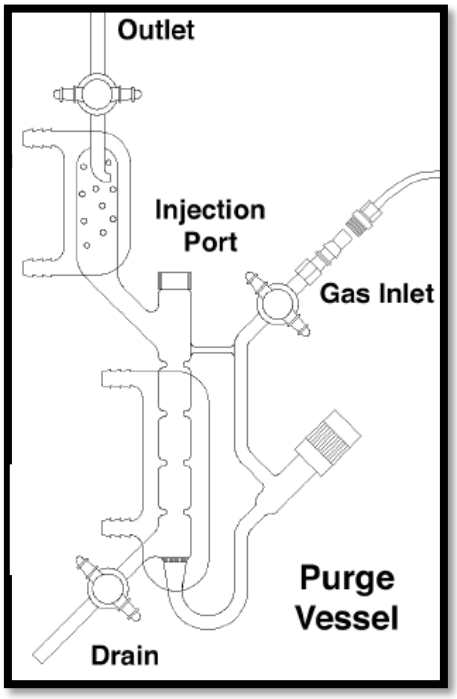


Figure 2.12 Purge vessel for the measurement of Nitric oxide in liquid samples.
Samples were injected into the purge that contains a reducing agent to convent nitrite to nitric oxide.

2.11.4 Nitrite measurement

A blank of 50 µl nitrite-free water was injected into the bottom of the purge vessel that contained the reducing agent. Next, 50 µl of each nitrite standard was injected and the peak areas were recorded. Then, 50 µl of each sample was injected and the peak areas were measured. A standard curve was drawn using the NO analysis programme and unknown concentrations determined accordingly.

2.12 Statistical analyses

In this thesis, all data are presented as mean ± SEM unless stated otherwise in the text. Data considering the effects of varying magnesium concentration on cell growth parameters, gene and protein expression were analysed using a one-way analysis of

variance (ANOVA; SPSS Statistics 22 software). Statistical significance between treatment groups was determined using Dunnett's test when variances were homogeneous among the tested groups. Games-Howell post-hoc analysis was used when the assumption of equal variances was not met. For these analyses the 1 mM $MgSO_4$ treatment was taken as the control group.

For experiments involving variation in magnesium conditions and stimulation with LPS or treatment with antibodies, Two-way analysis of variance was applied (General Linear Model; SPSS Statistics 22 software), followed by Dunntt's test if there is na univariate effect of magnesium in the absence of a magnesium × LPS interaction. Data with $p < 0.05$ were regarded as statistically significant. For all analyses, each treatment group comprised four samples (with a sample being single well on a culture plate). Any additional statistical tests used are provided in the methods section of each experimental chapter.

Figures were drawn using GraphPad Prism 6 software. Effects of varying magnesium concentration are shown as * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ compared to the 1 mM control.

3

Effect of Magnesium Concentration on HUVECs Proliferation

3 Effect of Magnesium Concentration on HUVECs Proliferation

3.1 Introduction

Endothelial cells are present along the length of all vessels, including both veins and arteries. They control the movement of macroparticles and hormones and regulate blood flow (Bouïs et al., 2001), cellular adhesion, vascular inflammation, vessel tone and smooth muscle proliferation (Deanfield et al., 2007). Endothelial dysfunction plays a role in many vascular diseases, such as thrombosis and atherosclerosis. Cultured endothelial cells have been used extensively as an *in vitro* model in pathological and physiological experiments relating to vascular disease (Bernardini et al., 2005).

Magnesium is fundamental to endothelial cell function (Ferrè et al., 2007). Several studies have drawn attention to the interaction of magnesium with ATP and its influence on the biochemical functions of nucleic acids, enzymes, phospholipids, and proteins (Wolf and Trapani, 2008, Ferrè et al., 2007, Bernardini et al., 2005, Maier, 2003). As magnesium is required for DNA, RNA, protein synthesis, and cell cycle regulation, it plays an important role in cell proliferation. The activation of some of the growth factor pathways, such as epidermal growth factor (EGF), require the formation of Mg-ATP complex, which makes magnesium a fundamental part of this process (De Baaij et al., 2015). Moreover, endothelial growth is impacted by magnesium deficiency through an increase in cytokine release in conditions of low magnesium concentration (Maier, 2003). The effect of magnesium on HUVECs proliferation and viability is due to the participation of magnesium in each cell cycle phase (Maier et al., 2004a).

In this chapter, the impact of magnesium deficiency on HUVECs proliferation was examined. The potential for magnesium to influence the endothelial cell response to an inflammatory challenge was also evaluated.

3.2 Hypothesis

The hypothesis was that magnesium deficiency inhibits endothelial cell proliferation.

3.3 Aim

The aim of this study was to investigate the impact of different concentrations of magnesium on HUVECs proliferation.

3.4 Objectives

This study sought to address three key research objectives:

1- Examine the effect of magnesium concentration on cell proliferation.

2- Evaluate whether high concentrations of magnesium in HUVECs cells can protect them from the effects of LPS-induced cell death.

3- To establish a protocol to enable investigation of the impact of magnesium upon endothelial cell function and responses to inflammatory signals.

3.5 Materials and Methods

The materials and methods used for these experiments are described in more detail in the Material and Methods chapter (Chapter 2).

3.5.1 Cell Culture

HUVECs were seeded in 96-well plates (for protein assay and trypan blue assays, a 6-well plate was used) and grown in EGM-2 with 2% FBS. The cells were then incubated at 37°C (5% CO_2) for 24 hours. The cells were then cultured in human endothelial, magnesium-free, medium (Invitrogen, USA), supplemented with low or high $MgSO_4$ concentrations (0.1 mM and 5 mM), compared to the physiological concentration of 1 mM $MgSO_4$. Cell growth was stopped at times 0, 72, and 120 hours. In experiments designed to test responses to an inflammatory challenge, HUVECs were grown in the presence of these varying $MgSO_4$ concentrations for three days and were then treated with LPS (0 µg/ml, 0.1 µg/ml, 0.5 µg/ml) for 4, 24, and 48 hours (see section 2.1.9).

3.5.2 Measurement of DNA Content in Cells

DNA was measured as a proxy for cell number and hence proliferation. To measure the amount of DNA in the HUVECs, seven dilutions of calf thymus DNA were used for the DNA standard curve. 100 µl of dye mixture was added to the wells that contain HUVECs, and the plate was scanned with a fluorescent plate reader (see section 2.2.2.1). The concentration of DNA in HUVECs was measured by comparing the fluorescent values to the standard curve.

3.5.3 Measurement of Total Protein Concentration

The Lowry assay was used to determine the amount of total protein in the cells. Dilutions of bovine serum albumin (BSA) were used for the standard curve. The Lowry solution and Folin reagents were prepared. Then, 50 µl of each BSA standard and 25 µl of the cell samples was added to 96-well plates followed by the addition of 150–175 µl of NaOH to each well. Lowry solution (50 µl) was added to all of the wells and, after 5 minutes, 50 µl of Folin reagent was added. The absorbance at 620 nm was read after 20 minutes using a microplate reader (see section 2.2.2.2).

3.5.4 MTT Assay

Confluent HUVECs were treated with different concentrations of magnesium for 0, 72, or 120 hours. Next, the media was replaced with HBSS containing 5 mg/ml MTT, and the cells were incubated for 4 hours at 37°C. The HBSS was removed and the formazan product was solubilized with DMSO. The absorbance of this product was measured at 590 nm to quantify the number of living cells (see section 2.2.3.1).

3.5.5 Trypan Blue Staining

HUVECs were cultured with varying concentrations of magnesium for three days. The cells were then treated with or without 0.1 µg/ml, or 0.5 µg/ml LPS for 4, 24, or 48 hours before staining with 0.04% trypan blue solution. The number of viable cells were then counted using a haemocytometer (see section 2.2.3.2).

3.6 Results

3.6.1 Effect of Magnesium Concentration on HUVEC Proliferation

3.6.1.1 DNA Content

To assess the effect of magnesium on HUVECs proliferation, DNA content was determined as described above. In an initial experiment, HUVECs were grown as per section 3.5.1, and their growth was stopped at three different times: 0, 72, and 120 hours. The second experiment stimulated HUVECs, with or without different LPS concentrations (0.1 µg/ml or 0.5 µg/ml), for 24 and 48 hours to examine the role of LPS on cell proliferation.

The results from the first experiment showed a significant time x concentration interaction (P=0.001, Figure 3.1). It appeared that there was an increase in the DNA content of the HUVECs after 120 hours of growth in 5 mM $MgSO_4$, relative to the DNA content of HUVECS grown in 1 mM at the same time point.

For the second experiment, as shown in Figure 3.2 A, Dunnett's test showed that in the presence of low magnesium, the DNA content decreased markedly after 24 hours in HUVECs grown in the presence of 0.5 µg/ml LPS (P=0.047) relative to the HUVECs grown in 1 mM $MgSO_4$. However the DNA content in HUVECs was not significantly affected by the presence of LPS or magnesium concentration for 48 hours (Figure 3.2 B).

Figure 3.3 shows light microscopy images of HUVECs cultured in different $MgSO_4$ concentrations for 96 and 120 hours. Figures 3.4 and 3.5 show microscopy images of HUVECs after stimulation with 0.1 µg/ml and 0.5 µg/ml LPS for 24 and 48 hours. There was a decrease in cell number in the 0.1 mM $MgSO_4$ groups treated with 0.1 µg/ml and 0.5 µg/ml LPS for both 24 and 48 hours, compared to the cells in the 5 mM $MgSO_4$ groups.

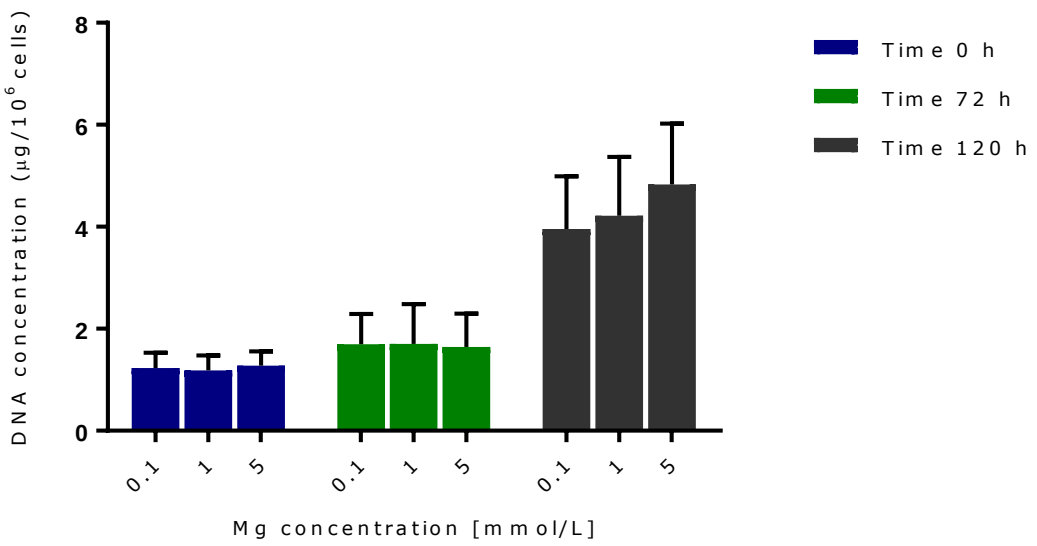


Figure 3.1 Concentration-dependent and time-dependent effects of $MgSO_4$ on the DNA content of cultured HUVECs. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for times 0, 72, and 120 hours. Data are shown as the mean ± SEM for n=4. Two-way ANOVA showed a significant interaction of magnesium concentration × time (P =0.001).

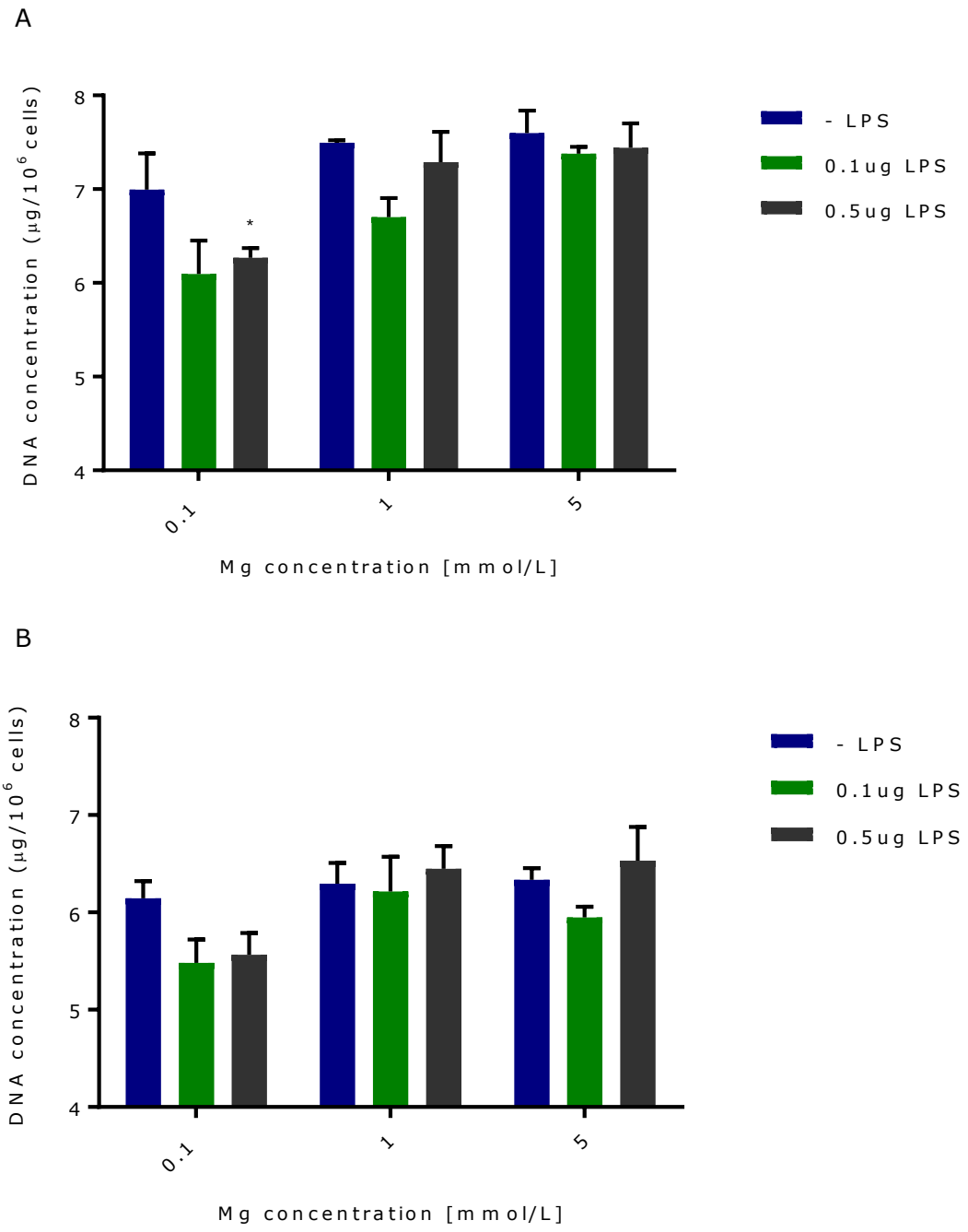
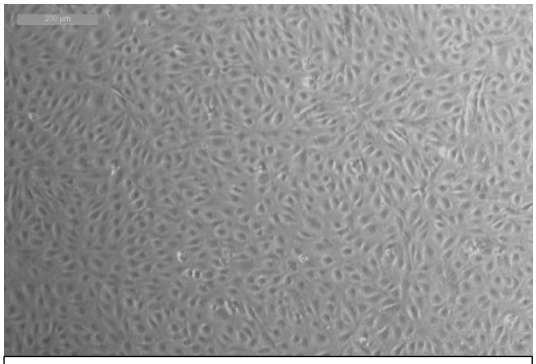
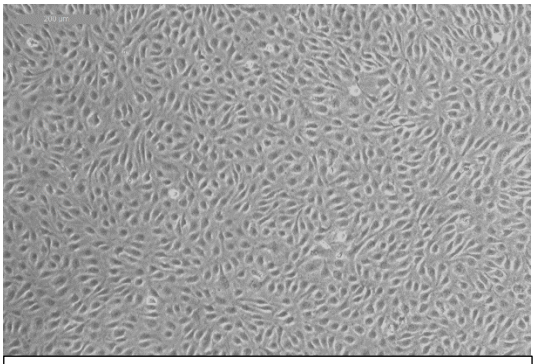


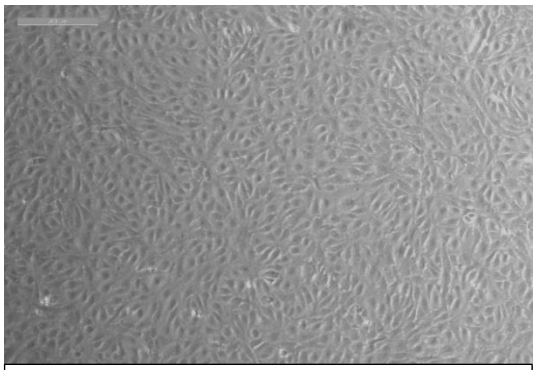
Figure 3.2. Concentration-dependent effects of $MgSO_4$ on the DNA content of HUVECs. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for three days followed by stimulation with or without 0.1 µg/ml, or 0.5 µg/ml LPS. Data are shown as mean ± SEM for n=4. A: HUVECs stimulated for 24 hours. Two-way ANOVA showed no significant interaction of magnesium concentration × LPS (P= 0.53). There was significant effect of magnesium concentration (P<0.001) and LPS (P= 0.019). * indicates P<0.05 compared to physiological 1 mM $MgSO_4$ in the same LPS treatment (Dunnett's test). B: HUVECs stimulated for 48 hours. Two-way ANOVA showed no interaction of magnesium concentration × LPS (P= 0.38). There was no significant effect of magnesium concentration or LPS.



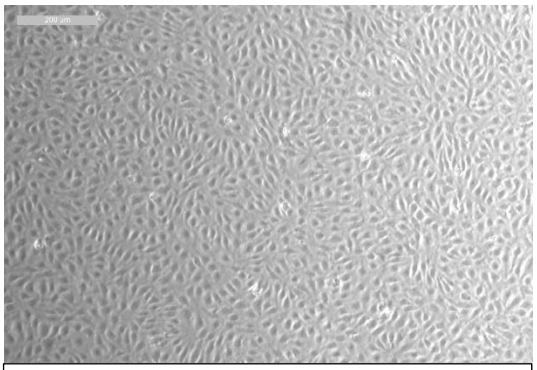
0.1 mM $MgSO_4$ for 96 hours



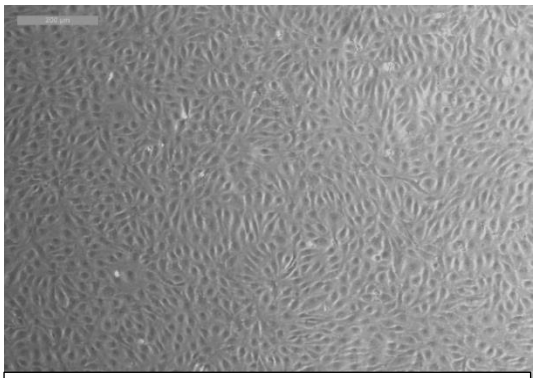
0.1 mM $MgSO_4$ for 120 hours



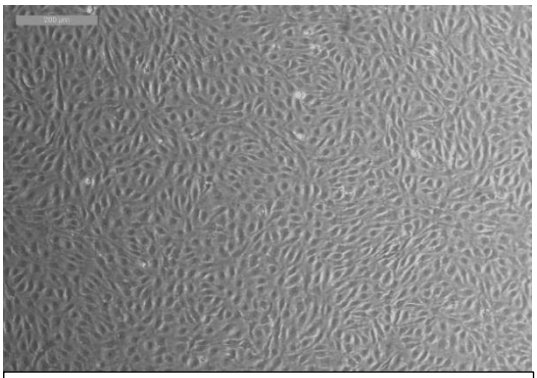
1 mM $MgSO_4$ for 96 hours



1 mM $MgSO_4$ for 120 hours

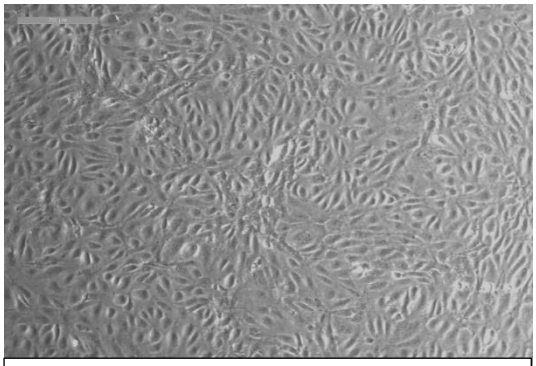


5 mM $MgSO_4$ for 96 hours

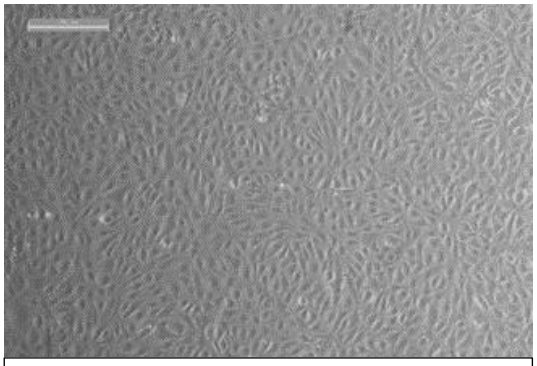


5 mM $MgSO_4$ for 120 hours

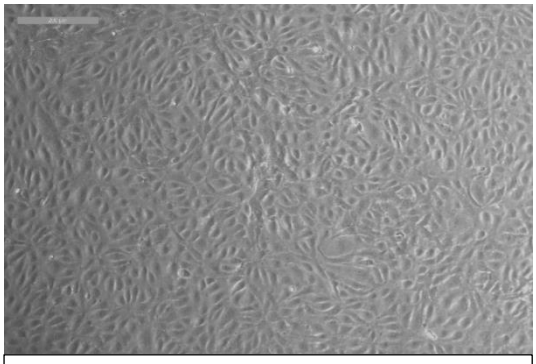
Figure 3.3 Light microscopy images illustrating the concentration-dependent effects of $MgSO_4$ on HUVECs. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for 96 and 120 hours.



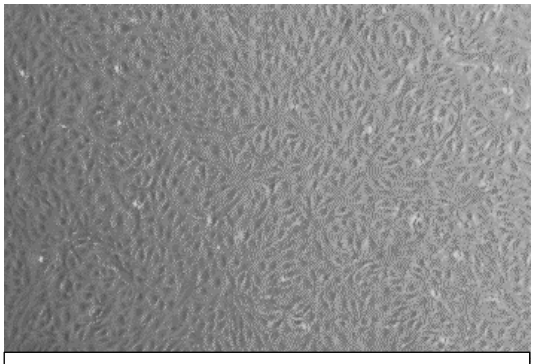
0.1 mM $MgSO_4$ with 0.1 µg LPS



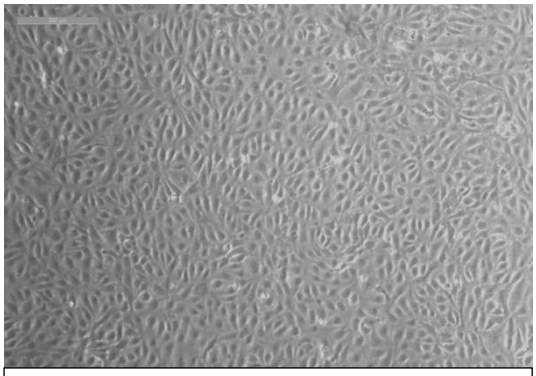
0.1 mM $MgSO_4$ with 0.5 µg LPS



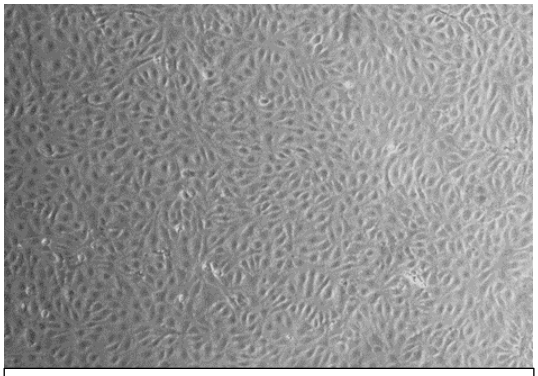
1 mM $MgSO_4$ with 0.1 µg LPS



1 mM $MgSO_4$ with 0.5 µg LPS



5 mM $MgSO_4$ with 0.1 µg LPS



5 mM $MgSO_4$ with 0.5 µg LPS

Figure 3.4 Light microscopy images illustrating the concentration-dependent effects of $MgSO_4$ on stimulated HUVECs. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for three days followed by stimulated with LPS (0.1 µg/ml or 0.5 µg/ml) for 24 hours.

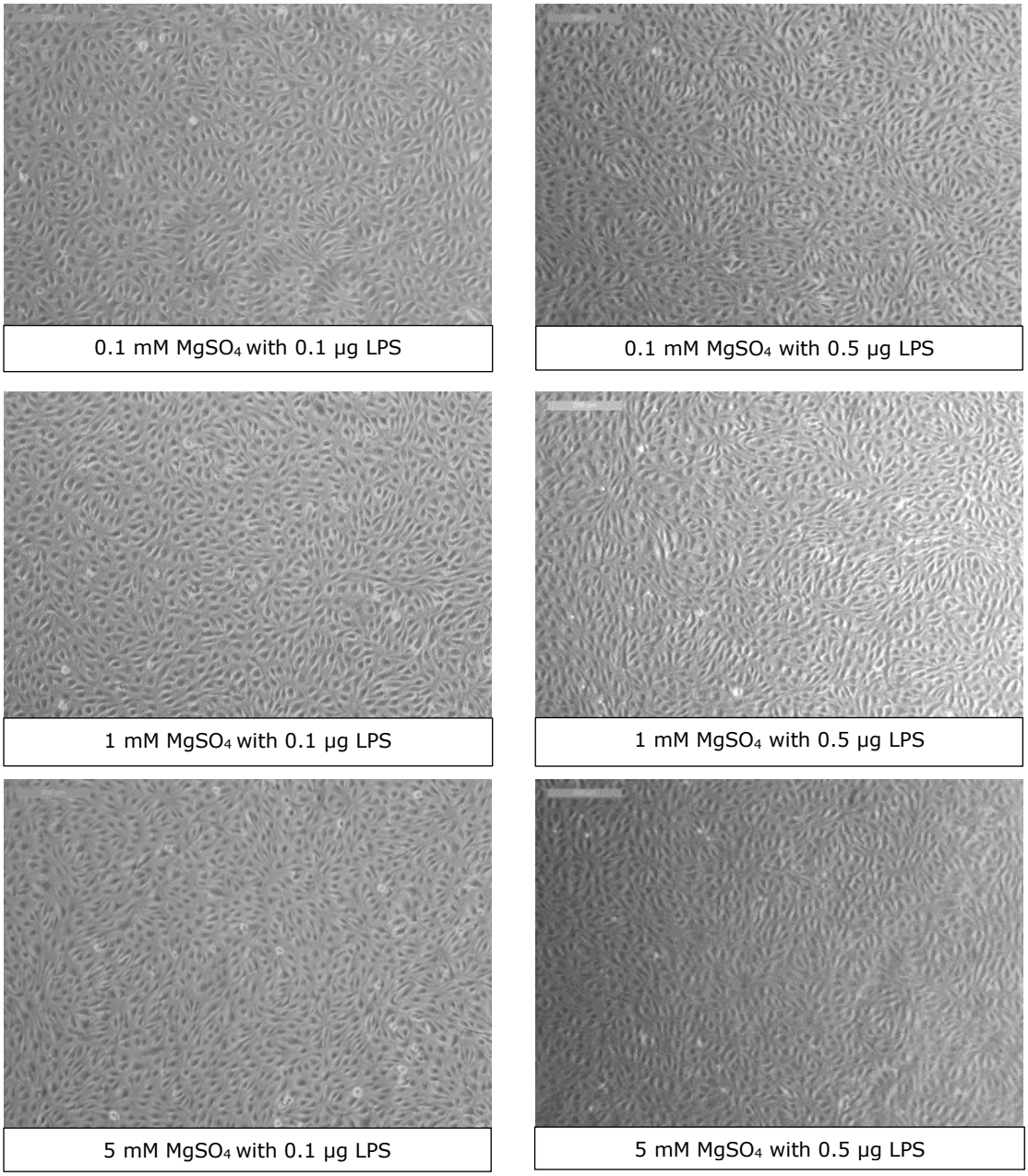


Figure 3.5 Light microscopy images illustrating the concentration-dependent effects of $MgSO_4$ on stimulated HUVECs. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for three days followed by stimulation with LPS (0.1 µg/ml or 0.5 µg/ml) for 48 hours.

3.6.1.2 Total Protein Content

As expected, the concentration of protein associated with cells increased with time in culture. However, significant interaction of magnesium concentration × time (P=0.003) were seen (Figure 3.6).

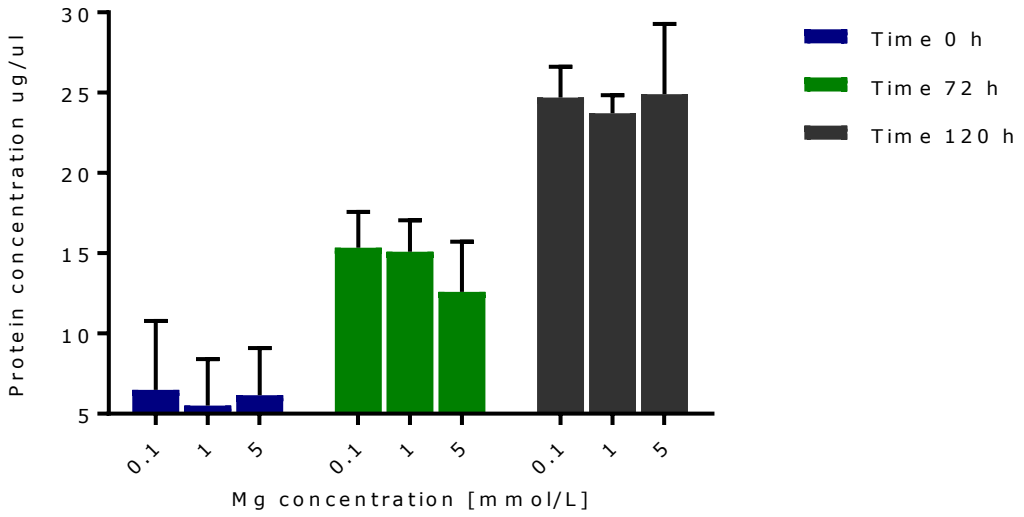


Figure 3.6 Concentration-dependent and time-dependent effects of $MgSO_4$ on the total protein concentration in HUVECs. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for times 0, 72, and 120 hours. Data are shown as mean ± SEM for n=4. Two-way ANOVA showed significant interaction of magnesium concentration × time (P= 0.003).

3.6.2 Effect of Magnesium Concentration on the Viability of HUVECs

3.6.2.1 MTT Assay

The MTT assay is a measurement of cell viability that is based on the premise that the mitochondria of living cells can reduce the tetrazolium salt MTT to a purple-coloured formazan product. When cells die, they lose the capability to change MTT into formazan. Therefore, colour formation serves as an indicator of live cells.

Figure 3.7 shows the cell viability of HUVECs cultured in various concentrations of $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for 72 hours and 120 hours. There was a significant interaction between magnesium concentration and time (P=0.034), which indicated that loss of viability only occurred with the longer incubation time. A small decrease in cell viability was seen after growth in 0.1 mM $MgSO_4$ for 120 hours when compared to the cells grown at 1 mM for the same time. No significant change was noted between the different treatment groups cultured for 72 hours.

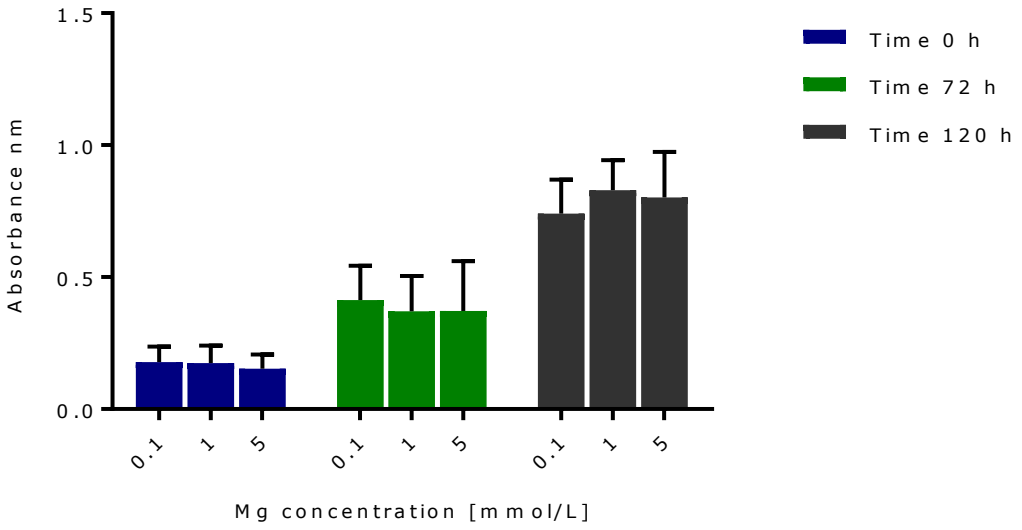


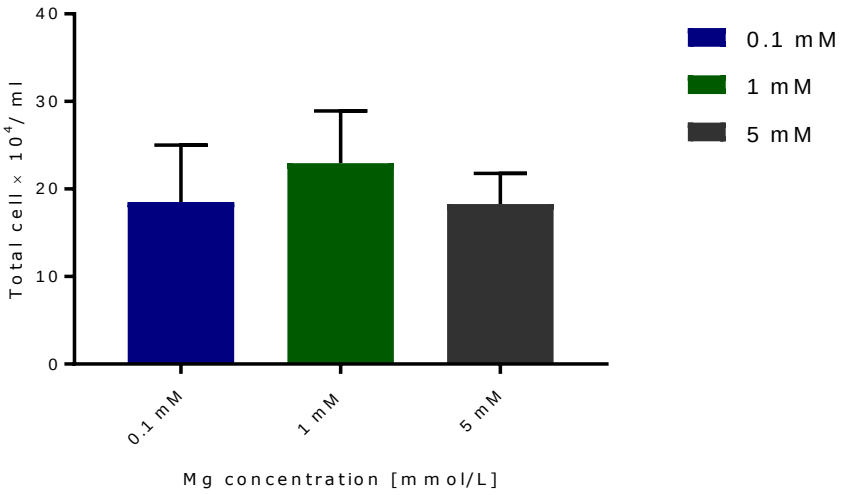
Figure 3.7 Concentration-dependent and time-dependent effects of $MgSO_4$ on HUVECs viability as determined by the MTT assay. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for times 0, 72, and 120 hours. Data are shown as mean ± SEM for n=4. Two-way ANOVA showed a significant interaction of magnesium concentration × time (P =0.034).

3.6.2.2 Trypan Blue Exclusion Assay

The trypan blue assay was also used to determine the number of viable cells in a cell suspension. HUVECs were cultured as described in section 3.5.1 and then exposed to 0.5 μg/ml LPS for 4hours, harvested with trypsin, and stained with trypan blue. The viable cells were counted using a haemocytometer. In some experiments, HUVECs were stimulated with 0 μg/ml, 0.1 μg/ml, or 0.5 μg/ml LPS for 24 and 48 hours.

Overall, HUVECs cultured in low magnesium concentrations were less viable, while those cultured in high magnesium concentrations had increased viability when compared to the control 1 mM (Figures 3.8 & 3.9). Specifically, HUVECs grown in low magnesium concentrations, and then stimulated with LPS for four hours, had significantly decreased in viability ($P<0.001$) relative to the control 1 mM (Figure 3.8). After HUVECs were stimulated with LPS for 24 hours, there was a significant interaction between magnesium concentration and LPS concentration ($P<0.001$). Likewise, HUVECs stimulated with 0.1 μg/ml LPS and with 0.5 μg/ml LPS for 48 hours had a significant interaction between the magnesium concentration and the LPS concentration ($P=0.018$). Cells grown in 5 mM $MgSO_4$ showed greater viability after treatment with 0.5 μg/ml LPS for 24 and 48 hours, relative to the control 1 mM. Conversely, treatment of HUVECs with 0.1 μg/ml or 0.5 μg/ml LPS for 24 or 48 hours showed decreased cell viability when cultured in 0.1 mM $MgSO_4$ (Figures 3.9 A and 3.9 B).

A



B

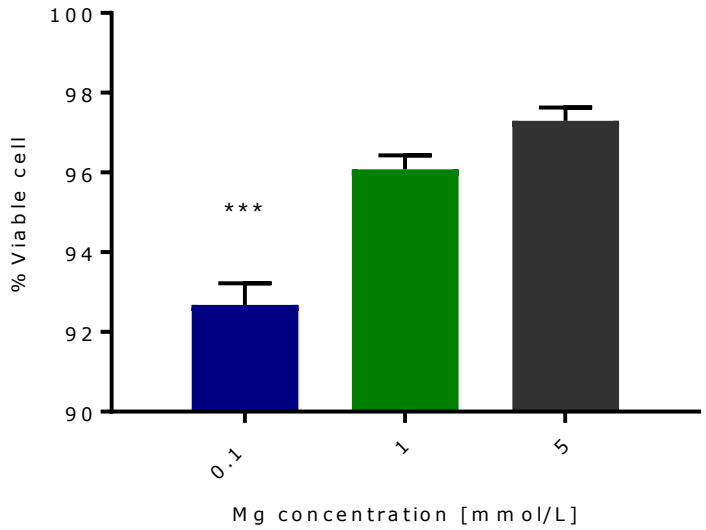


Figure 3.8 Dependence of HUVECs viability on $MgSO_4$ concentration as determined by the trypan blue exclusion assay. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for three days followed by stimulation with LPS (0.5 µg/ml) for four hours. Data are shown as mean ± SEM for n=4. A: Total cell number. B: Viable cell stimulation with LPS for 4 hours. One way ANOVA showed significant effect of magnesium concentration on cell viability ($P< 0.001$). *** indicates $P<0.001$ compared to physiological 1 mM $MgSO_4$ (Dunnett's test).

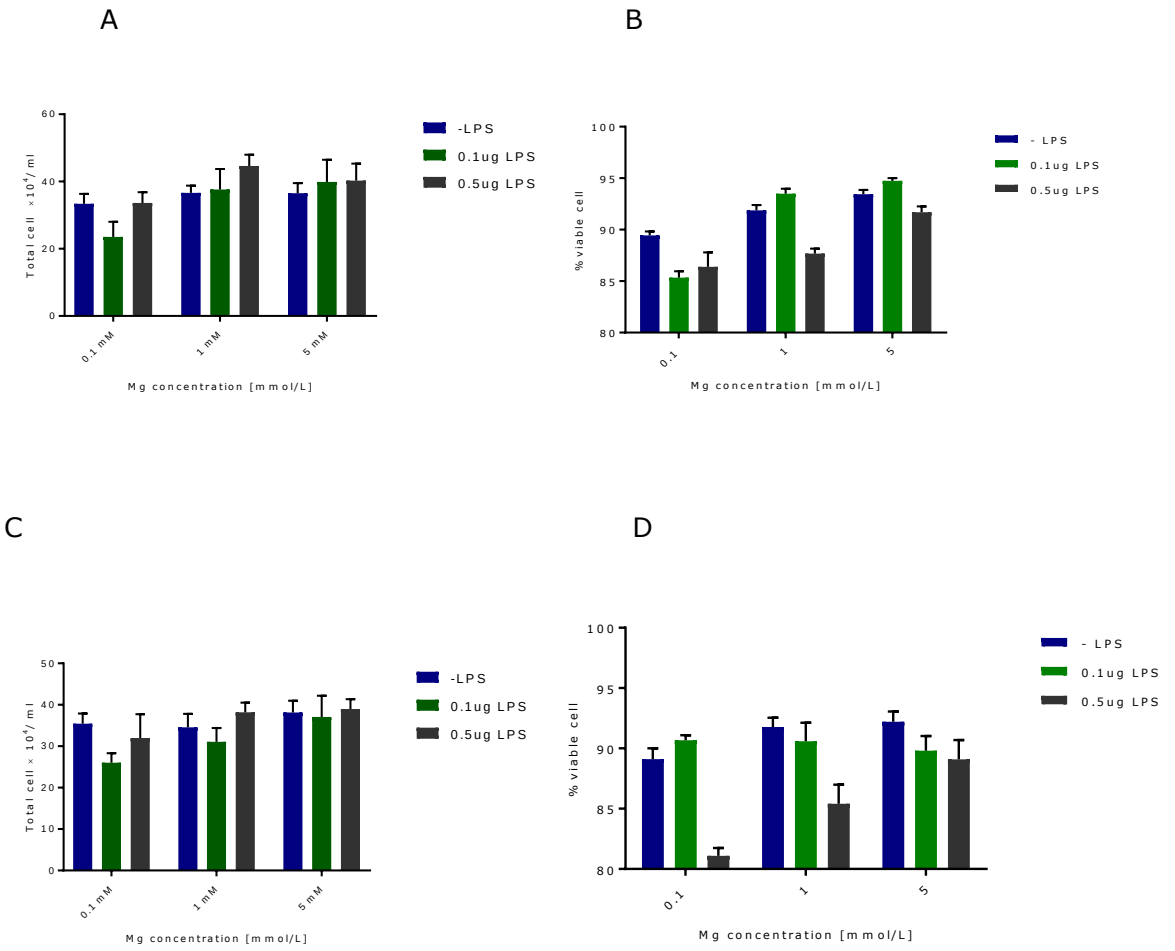


Figure 3.9 Dependence of HUVECs viability on $MgSO_4$ concentration as determined by the trypan blue exclusion assay. HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM, and 5 mM) for three days followed by stimulation with or without LPS (0.1 µg/ml, or 0.5 µg/ml). Data are shown as mean ± SEM for n=4. A: Total cells after 24h. B: Viable cells stimulation after 24 hours. Two-way ANOVA showed a significant interaction of magnesium concentration × LPS concentration (P <0.001). C: Total cells after 48h. D: Viable cells after stimulation for 48 hours. Two-way ANOVA showed a significant interaction of magnesium concentration × LPS concentration (P <0.018).

3.7 Discussion

Magnesium is reported to affect endothelial cell proliferation due to its important role in angiogenesis, metabolic activity, and DNA/RNA synthesis (Swaminathan, 2003, Chakraborti et al., 2002). In the present study, HUVECs were cultured in various magnesium concentrations for different amounts of time in order to study changes in cell proliferation. Even slight alterations in the magnesium concentration can lead to changes in the signalling pathways that control vascular functions and regulate cell proliferation (Sontia and Touyz, 2007). This study was designed to monitor proliferation, as an outcome of these alterations. Of the concentrations that were used, 0.1 mM was considered to be in the deficiency range and 5 mM was a high concentration, while 1 mM served as the physiological control (Ferrè et al., 2007, Maier et al., 2004b). This study ran three tests to quantify the cell proliferation and viability with each test measuring different parameters. DNA assay indicates the cell number, trypan blue determines the number of dead cells, while the MTT assay measures the metabolic activity in the cell mitochondria (Quent et al., 2010). Overall, the DNA assay has been suggested to represent the most sensitive method (Quent et al., 2010, Wang et al., 2010).

The present study showed that HUVECs cultured in a low magnesium concentration did not proliferate as rapidly as cells grown in the normal magnesium range (1 mM), and that cell viability was lower as measured by the DNA, MTT and trypan blue assays. This was consistent with the study hypothesis.

Furthermore, these results showed that low magnesium concentrations significantly decreased the viability of LPS stimulated HUVECs as measured by DNA content and trypan blue assays. Our results are in agreement with many studies that have also found a decreased proliferation of HUVECs after culturing in low magnesium concentrations (Ferrè et al., 2007, Maier et al., 2004b, Banai et al., 1990).

LPS, which is a part of the gram negative bacteria membrane (Andreakos et al., 2004), can activate many inflammatory responses in the host cells (Yang et al., 2007). LPS was used to subject the cells to an inflammatory challenge (Tam et al., 2011), relying on the

observation that LPS results in cell dysfunction (Faure et al., 2001). In magnesium deficient cells, reduced cell viability was seen at both concentrations of LPS that were used. In contrast, the high magnesium concentration protected the HUVECs from LPS induced cell death, when stimulated with 0.5 µg/ml LPS for 24 and 48 hours. Further, HUVECs proliferation in the high magnesium concentration for 120 hours increased relative to the physiological concentration, as measured by the DNA content assay. The data are consistent with other studies that indicate that a high magnesium concentration increases the proliferation of HUVECs (Bernardini et al., 2005, Maier et al., 2004a).

Low magnesium levels are suggested to impact upon HUVECs proliferation by several mechanisms, including decreasing the amount of Mg-conjugated ATP and by controlling the amount of cyclic AMP present in the cell (Banai et al., 1990). Magnesium can modulate cell proliferation through DNA transcription, RNA translation, DNA duplication and protein synthesis (Rubin, 2007). It is also required for the catalysis of many enzymes that are important in the proliferation process, such as polymerases, topoisomerases and endonucleases (Wolf and Cittadini, 1999). An increase in cytokine production in low magnesium concentrations can affect the growth of HUVECs (Bernardini et al., 2005).

Magnesium also has an important role in the regulation of the cell cycle (Wolf and Trapani, 2008). Low magnesium leads to a rise in the number of cells in G0/G1 and a decline in the number of cells in S phase. This modulation is likely to occur through increased expression of the cell cycle inhibitors p21 and p27 (Wolf et al., 2008a). Likewise, the extracellular magnesium concentration affects cyclin D, which activates cyclin-dependent kinases. These kinases regulate the G phase, which inhibits the retinoblastoma (Rb) regulation restriction point (Wolf and Trapani, 2008). In addition, low magnesium levels decrease the expression of the phosphatase CDC25B, which regulates the progression from G2 to M phase in the cell cycle (Maier et al., 2004b).

The findings presented in this chapter confirm previously reported effects of magnesium on the proliferation of endothelial cells in culture. Although confirmatory, these observations are important to verify the utility of a working protocol to prove our

hypothesis, and a good starting point for investigation of endothelial cell function with varying magnesium concentrations.

The next step for the project was to carry out an evaluation of the effect of magnesium on the endothelial cell transcriptome, using a gene microarray. This is a powerful tool to evaluate the mechanisms which may be sensitive to magnesium concentration and which are relevant to cardiovascular function and disease.

3.8 Conclusion

The data from these initial *in vitro* experiments indicate that low magnesium levels can decrease endothelial cell proliferation and viability and that this decrease is even more striking in the presence of LPS. In contrast, HUVECs proliferation increases when cells are cultured with high magnesium levels for five days. Magnesium treatment also protects the HUVECs from LPS induced cell death. Low magnesium levels potentially influence many possible biochemical pathways that can affect HUVECs proliferation. Although this study did not directly assess atherosclerosis, the findings support the general view that the effects of magnesium in cell growth and proliferation may be important for preventing atherosclerosis by healing vascular injuries which can contribute to disease development, or offsetting the negative effects of inflammation.

4

Effects of Magnesium on Gene Expression in HUVECs

4 Effects of Magnesium on Gene Expression in HUVECs

4.1 Introduction

Mg^{2+} ions are fundamental to various cell functions due to their role in DNA and protein synthesis. They are also an essential cofactor for numerous enzymes, most of which are involved in cellular metabolism, muscle contraction, the density of bone and stabilizing the structures of cell membranes, nucleic acids and proteins (De Baaij et al., 2015, Yang et al., 2004).

Work presented in the previous chapter indicated that varying the magnesium concentration in cell culture medium, influenced the proliferation of cultured vascular endothelial cells and their response to an inflammatory challenge. Magnesium controls vascular tone by stimulating the response to vasodilators and vasoconstrictors. Moreover, magnesium controls cell growth and can protect vascular cells from oxidative stress due to its antioxidant and anti-inflammatory functions. Clinical studies indicate that magnesium deficiency is implicated in cardiovascular disease, atherosclerosis, diabetes and metabolic syndrome (Champagne, 2008, Wolf et al., 2008a, Sontia and Touyz, 2007, Maier, 2003). As such, the findings from the previous chapter are consistent with literature linking magnesium status to cardiovascular function.

The endothelium is a layer of cells that lines the interior surface of all blood vessels (Sader and Celermajer, 2002). It plays a key role in controlling vascular homeostasis by releasing paracrine factors that influence vascular tone, such as endothelium-induced relaxing factors and endothelium-derived contracting factors, which preserve the vascular tissue from thrombosis and atherosclerosis (Kietadisorn et al., 2012, Förstermann and Münzel, 2006). Evidence has shown that endothelial dysfunction is associated with a low extracellular magnesium concentration, potentially through its effects on cell proliferation, the production of nitric oxide and calcium release, all of which are involved in the underlying pathology of many cardiovascular diseases (Maier et al., 2004a, Maier, 2003). Whilst such effects of magnesium on the vascular endothelium are well-documented, less

is known about the mechanisms through which magnesium impacts upon endothelial cell function.

4.2 Aim

The results from the previous chapter have shown that magnesium concentrations can affect the proliferative capacity of HUVECs. To understand the mechanisms which underpin these effects of magnesium on endothelial cells, this study aims to focus on two crucial elements of endothelial function; the production of nitric oxide and endothelin and cation transport. In addition, it aims to look at the effect of varying magnesium concentrations on the full transcriptome of HUVECs, in order to gain insights into the mechanisms underlying its effects.

4.3 Hypothesis

The hypotheses for this study are that the function and protein and gene expression of cultured endothelial cells are regulated by magnesium concentration, and magnesium regulates endothelial cell function through regulating nitric oxide production; endothelin production and expression of other genes which remain to be identified.

4.4 Objectives

1. Identify the genes, proteins, pathways and regulatory networks in HUVECs culture that are responsive to varying concentrations of magnesium sulphate.

2. Evaluate whether various concentrations of magnesium sulphate affect nitric oxide secretion by HUVECs.

3. Determine the impact of various concentrations of magnesium sulphate on endothelin production by HUVECs.

These objectives were addressed through two approaches. Firstly, a microarray analysis was conducted to identify potential gene targets for future analysis. The second approach was a candidate gene approach, which sought to examine the expression of genes (mRNA) and proteins that are known from the literature to be involved in magnesium functions in the cell, or aspects of vascular endothelial cell function.

4.5 Materials and Methods

The materials and methods are explained in detail in the Material and Methods chapter (Chapter 2).

4.5.1 Cell Culture

HUVECs were cultured in six-well plates with 2% FBS until they reached 80% confluence. Then, the HUVECs were cultured in human endothelial magnesium-free medium (Invitrogen, USA) supplemented with $MgSO_4$ at concentrations of 0.1 (low), 1 (control) or 5 (high) mM. The cultures were stopped at 24, 72 and 120 hours for endpoint measurements. In some of the experiments, the cells were treated with LPS (0.5 µg/ml) for 4 hours, and the response was compared to untreated cells (see Section 2.1.9).

4.5.2 Measurement of Gene Expression by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For qRT-PCR analysis, the total RNA was isolated using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. First-stranded cDNA synthesis was performed using a ReverseAid RT Kit (Thermo). qRT-PCR was performed using a Roche LightCycler 480 and SYBR Green Master Mix. All genes were normalised to the cyclophilin housekeeping gene (see Section 2.3), which did not change expression in response to magnesium concentration or LPS treatment.

4.5.3 Microarray Analysis

Microarray analysis was conducted at Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, School of Biosciences using a GeneChip® Human Genome U133 Plus 2.0 Array, GeneChip® Hybridization, Wash, and Stain Kit and a GeneChip® 3'IVT PLUS Reagent Kit (Affymetrix, High Wycombe, Bucks, UK) according to the manufacturer's protocol (see Section 2.4).

4.5.3.1 Bioinformatics Analysis

The microarray experiment included three samples for each group. The arrays were scanned by an Affymetrix Scanner 3000 7G System. Affymetrix CEL files were imported into Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). The Quality Control (QC) metrics were checked by examining surface defects, hybridization, labelling, and a

ratio of the 3′ probe set to the 5′ probe set (3′/5′ ratio) to provide the quality of the microarray data. The values were $\log^2$ transformed and quantile normalisation using the Robust Multi-array Average (RMA). The results were considered significant when $p < 0.05$ was identified by one-way ANOVA. To analyse the effects of magnesium treatment, the high and low groups were compared to the physiological concentration, 1 mM.

4.5.4 Western Blot Analysis

After the cells were lysed, protein samples were denatured and then 30 µg of the protein samples loaded onto 4-10% mini-protean TGX precast polyacrylamide gradient gel. The seperated proteins were transferred to a nitrocellulose membrane, and then, the membrane was blocked. The proteins were detected using primary antibodies (anti-TRPM-7 (Abcam) and anti-eNOS3 (Santa Cruz)). Next, a secondary antibody, horseradish peroxidase (HRP)-labelled anti-rabbit monoclonal, was applied to detect the primary antibody. The membranes were then incubated with enhanced chemiluminescence (ECL) detection reagent (Prime Western Blotting Detection Reagent Kit, GE Healthcare) and visualised via X-ray film exposure (see Section 2.5).

4.5.5 Nitric Oxide Production Measurement

The cell culture media were collected to measure the nitric oxide (NO) concentration using a Sievers Nitric Oxide Analyzer (NOA 280i).

4.6 Results

4.6.1 Microarray approach: Effect of Magnesium Concentration on the HUVECs transcriptome

The effects of magnesium on mRNA expression were initially investigated using microarray analysis. RNA was extracted from HUVECs cultured in various magnesium concentrations (0.1 mM, 1 mM, and 5 mM) for 24 hours. To determine if the treatments differentially affected the various groups of genes, the microarray data, was initially examined using principal component analysis (PCA). Figure 4.1 shows a clear separation between the various groups. They were clustered separately from one another in terms of mRNA expression in each group, indicating that there were large effects of the different treatments and these effects did not overlap. In each group the sample intensity values were close together suggesting that the mRNA expression was well conserved within each group (i.e. low variance).

The microarray results demonstrated that 5,758 transcripts out of approximately 47,000 transcripts were differentially expressed in the high- and the low-magnesium treatments as compared to the control. The Venn diagram below (Figure 4.2) shows that 2,728 genes were differentially expressed after the HUVECs were cultured in 0.1 mM $MgSO_4$, and 1,434 of these genes were down-regulated and 1,294 were up-regulated. HUVECs cultured in 5 mM $MgSO_4$ expressed 3,030 genes differently to the control, with 1,394 genes being down-regulated and 1,636 genes being up-regulated. Tables 4.1 and 4.2 contain a list of the top 20 genes that were differentially expressed in HUVECs cultured in 0.1 and 5 mM $MgSO_4$ based on Log2 fold change. The full dataset from both treatments are available as a file on the disc submitted with this thesis as supplementary material. Six hundred and fifteen genes were differentially expressed in both the high- and low-magnesium treatments relative to the control.

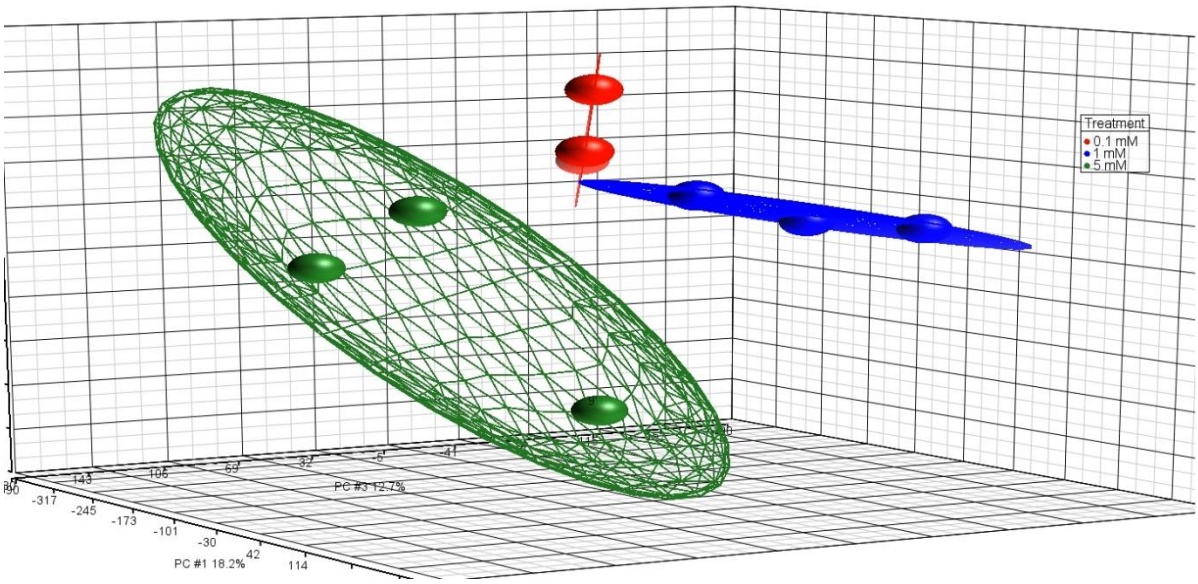


Figure 4.1 Three-dimensional PCA plot for the microarray data. Each point represents a chip. The red indicates the 0.1 mM $MgSO_4$ group. The blue indicates the 1 mM $MgSO_4$ group. The green indicates the 5 mM $MgSO_4$ group. The axis in a PCA plot are unit-less. The numbers in each axis are the raw component scores. These are derived as orthogonal linear combinations of the variables in the data set. The PCA plot is an exploratory technique that describes the structure of high dimensional data by reducing its dimensionality.

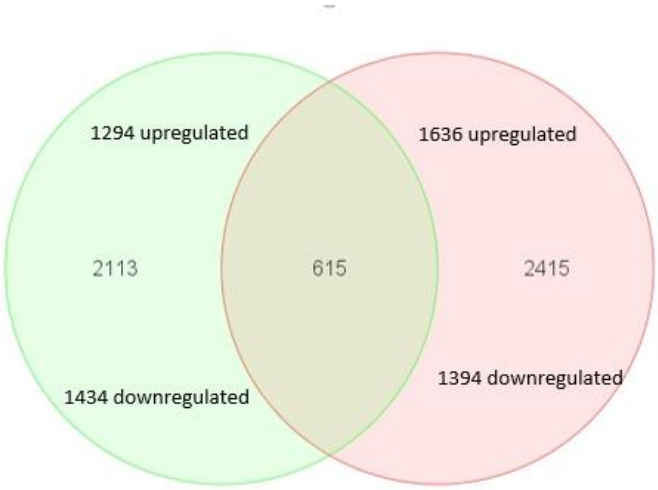


Figure 4.2 Venn diagram of HUVECs genes that were significantly expressed after culturing for 24 h in 0.1 mM $MgSO_4$, indicated by green, and 5 mM $MgSO_4$, indicated by red, as compared to 1 mM $MgSO_4$. 615 genes were differentially expressed relative to control in both conditions.

Table 4.1 List of top 20 genes based on fold change that were differentially expressed in HUVECs culture in 0.1 mM $MgSO_4$

Symbol	Gene Title	*P*-value	Log 2 Fold-Change	Fold-Change Description
MDM2	Mdm2 p53 binding protein homolog (mouse)	0.046	2.1	0.1 mM up vs 1 mM
FUS	Fused in sarcoma	0.031	1.7	0.1 mM up vs 1 mM
ARSD	Arylsulfatase D	0.038	1.7	0.1 mM up vs 1 mM
NPIPL3	Nuclear pore complex interacting protein-like 3	0.031	1.7	0.1 mM up vs 1 mM
SRSF11	Serine/arginine-rich splicing factor 11	0.046	1.6	0.1 mM up vs 1 mM
RBM5	RNA binding motif protein 5	0.021	1.5	0.1 mM up vs 1 mM
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	0.003	1.5	0.1 mM up vs 1 mM
LOC100507226	Hypothetical LOC100507226	0.04	1.5	0.1 mM up vs 1 mM
ZNF224	Zinc finger protein 224	0.008	1.4	0.1 mM up vs 1 mM
PSMB7	Proteasome subunit, beta type, 7	0.003	1.4	0.1 mM up vs 1 mM
ERGIC1	Endoplasmic reticulum-golgi intermediate compartment (ERGIC) 1	0.045	-1.7	0.1 mM down vs 1 mM
ATF6B	Activating transcription factor 6 beta	0.045	-1.7	0.1 mM down vs 1 mM
UGGT2	UDP-glucose glycoprotein glucosyltransferase 2	0.001	-1.6	0.1 mM down vs 1 mM
RNF6	Ring finger protein (C3H2C3 type) 6	0.023	-1.5	0.1 mM down vs 1 mM
HOXB8	Homeobox B8	0.024	-1.5	0.1 mM down vs 1 mM
CHD9	Chromodomain helicase DNA binding protein 9	0.019	-1.5	0.1 mM down vs 1 mM
CTDSPL2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like	0.042	-1.5	0.1 mM down vs 1 mM
RBBP9	Retinoblastoma binding protein 9	0.001	-1.4	0.1 mM down vs 1 mM
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	0.042	-1.4	0.1 mM down vs 1 mM
PGGT1B	Protein geranylgeranyltransferase type I, beta subunit	0.001	-1.4	0.1 mM down vs 1 mM

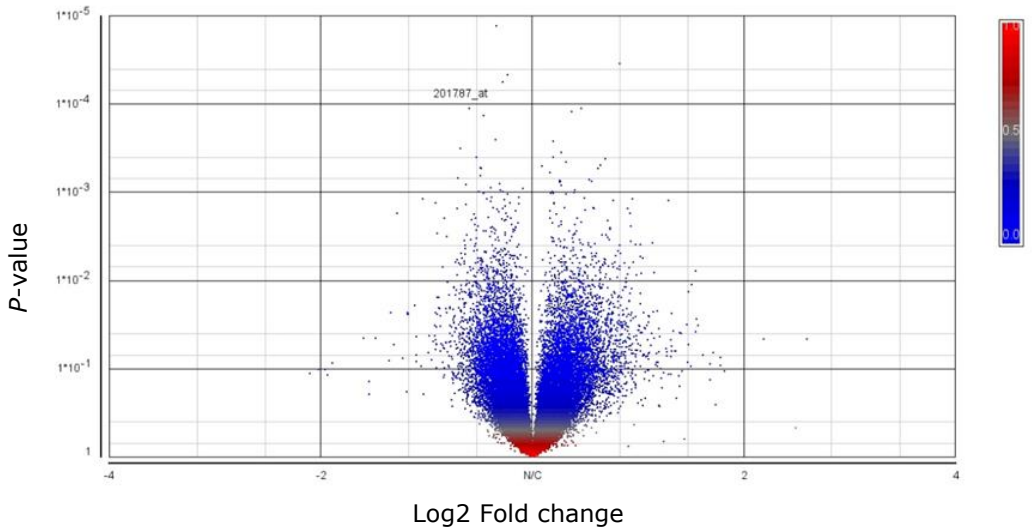
Table 4.2 List of top 20 genes based on fold change that were differentially expressed in HUVECs culture in 5mM $MgSO_4$

Gene Symbol	Gene Title	*P*-value	Log2 Fold-Change	Fold-Change Description
PROX1	Prospero homeobox 1	0.001	1.6	5 mM up vs 1 mM
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	0.01	1.5	5 mM up vs 1 mM
SLC12A4	Solute carrier family 12 (potassium/chloride transporters), member 4	0.034	1.5	5 mM up vs 1 mM
JMY	Junction mediating and regulatory protein, p53 cofactor	0.04	1.4	5 mM up vs 1 mM
ZFHX3	Zinc finger homeobox 3	0.002	1.4	5 mM up vs 1 mM
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	0.018	1.4	5 mM up vs 1 mM
TFRC	Transferrin receptor (p90, CD71)	*P*<0.001	1.4	5 mM up vs 1 mM
SMPX	Small muscle protein, X-linked	0.004	1.4	5 mM up vs 1 mM
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	0.018	1.4	5 mM up vs 1 mM
TSC22D2	TSC22 domain family, member 2	0.038	1.3	5 mM up vs 1 mM
GALNTL2	UDP-N-acetyl-alpha-D-galactosamine	*P*<0.001	-2.0	5 mM down vs 1 mM
FILIP1	Filamin A interacting protein 1	*P*<0.001	-1.9	5 mM down vs 1 mM
CXCL6	Chemokine (C-X-C motif) ligand 6	*P*<0.001	-1.8	5 mM down vs 1 mM
USP9X	Ubiquitin specific peptidase 9, X-linked	0.039	-1.7	5 mM down vs 1 mM
CLASP1	Cytoplasmic linker associated protein 1	0.007	-1.6	5 mM down vs 1 mM
SCG5	Secretogranin V	0.004	-1.6	5 mM down vs 1 mM
FILIP1	Filamin A interacting protein 1	*P*<0.001	-1.6	5 mM down vs 1 mM
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	0.004	-1.5	5 mM down vs 1 mM
AQP1	Aquaporin 1 (Colton blood group)	*P*<0.001	-1.5	5 mM down vs 1 mM
IL8	Interleukin 8	0.011	-1.5	5 mM down vs 1 mM

Figure 4.3 shows the Volcano plot for the genes. In the plot, The X-axis indicates the fold change and the Y-axis indicates the *p* value. Each dot represents a gene. The transcripts down-regulated are on the left of the plot, and the transcripts that are up-regulated are on the right side. The plot shows that transcripts were differentially regulated by low or high magnesium concentrations, the fold-changes were generally low almost all transcripts expressed within the Log2 fold-change range (-2 to 2).

Before examining in detail which genes, processes and pathways were differentially expressed in low- or high-magnesium conditions, a sub-analysis was conducted to assess the 615 transcripts that were differentially expressed in both conditions, Tables 4.3 and 4.4 show a list of top 20 genes based on Log2 fold change of the high magnesium treatments that significantly up and down-expression from the genes that differentially expressed in both the high- and low-magnesium treatments relative to the control. Figure 4.4 shows the hierarchical cluster analysis (heat map) of these 615 transcripts. Each gene is represented in one column, and each sample is represented in one row. The heat map revealed distinctly different expression patterns between the control (1 mM $MgSO_4$) and the high- and low-magnesium treatment groups. As is clear from the heat map, most of these transcripts (581 transcripts) were up- or down-regulated in both low- and high-magnesium conditions. As this was an unexpected outcome, the Gene Ontology (GO) Consortium bioinformatics resource (GOC, http://www.geneontology.org) was used to classify and describe functions of the common genes that were differentially expressed in high- and low-magnesium conditions. This analysis showed that the two conditions had effects consistent with disruption of the cytoskeleton, intracellular membrane bound organelles, and protein and anion binding.

A



B

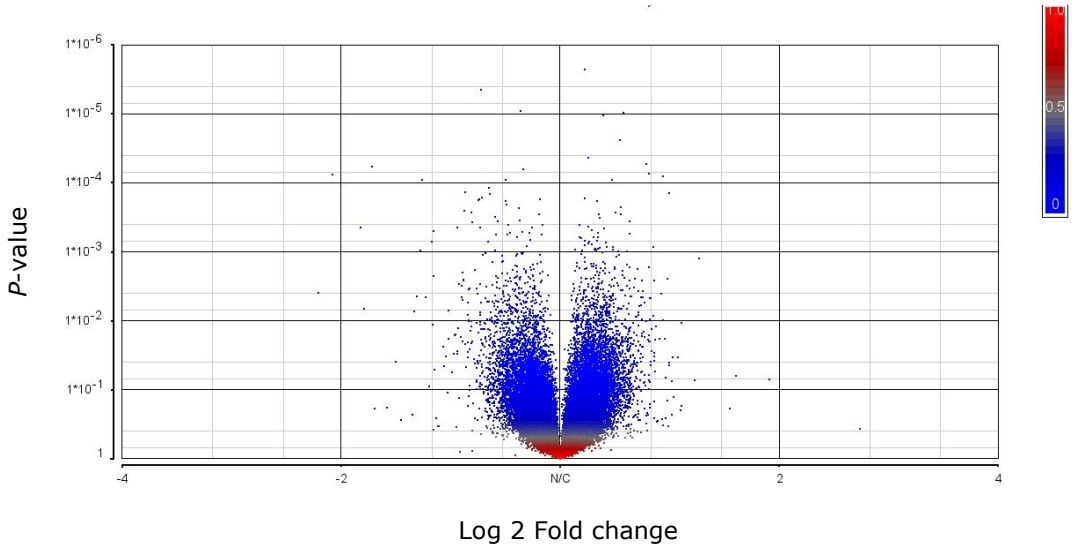


Figure 4.3 Volcano plot representing the microarray results. A: 0.1 mM $MgSO_4$ group. B: 5 mM $MgSO_4$ group. Each dot represents one gene. The X-axis represents fold change, and the Y-axis represents the *p* value. The blue dots indicate more statistically significant. Genes located in the top left corner are highly significantly down-regulated, while the genes in the top right corner are highly significantly up-regulated.

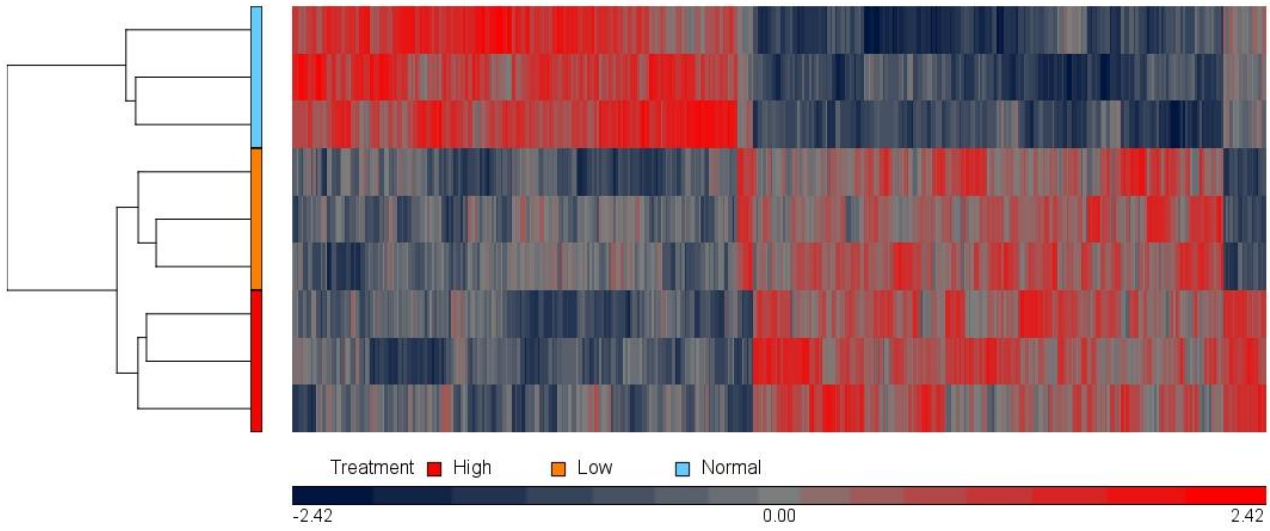


Figure 4.4 Hierarchical clustering of the 615 genes that were commonly expressed in high- and low-magnesium treatments, with blue blocks representing down-regulated expression and red blocks representing up-regulated expression. Each row represents one sample, and each column represents one gene. The red bar on the side of the heat map represents the 5 mM $MgSO_4$ group, n = 3. The blue bar represents the 1 mM $MgSO_4$ group, n = 3. The orange bar represents the 0.1 mM $MgSO_4$ group. The scale at the bottom of the diagram represents Log2 fold-change values.

Table 4.3 List of top 20 genes based on Log2 fold change of the high magnesium treatments that were significantly up-regulated by both the high- and low-magnesium treatments relative to the control

Gene Symbol	Gene Title	5 mM vs 1 mM		0.1 mM vs 1 mM	
		p-value	Log2 Fold-Change	*p*-value	Log2 Fold-Change
PROX1	Prospero homeobox 1	0.001	1.6	0.032	1.2
ZFHX3	Zinc finger homeobox 3	0.002	1.4	0.017	1.2
DCTN1	Dynactin 1	0.002	1.3	0.009	1.2
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	0.004	1.3	0.006	1.3
GRINA	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1	*P*<0.001	1.3	*P*<0.001	1.2
PTPRF	Protein tyrosine phosphatase, receptor type, F	0.005	1.3	0.026	1.2
CEP68	Centrosomal protein 68kDa	0.016	1.3	0.015	1.3
OR10D1P	Olfactory receptor, family 10, subfamily D, member 1 pseudogene	0.008	1.3	0.029	1.2
BNC2	Basonuclin 2	0.003	1.3	0.003	1.3
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide	0.007	1.3	0.014	1.2
FAM149A	Family with sequence similarity 149, member A	0.016	1.3	0.035	1.2
CCDC81	Coiled-coil domain containing 81	0.032	1.3	0.042	1.2
LOC100240734	Hypothetical LOC100240734	0.004	1.3	0.017	1.2
C22orf26	Chromosome 22 open reading frame 26	0.012	1.2	0.011	1.3
KIAA2026	KIAA2026	0.012	1.2	0.048	1.2
KLF6	Kruppel-like factor 6	0.013	1.2	0.032	1.2
MACF1	Microtubule-actin crosslinking factor 1	0.020	1.2	0.008	1.3
ACADL	Acyl-CoA dehydrogenase, long chain	0.01	1.2	0.024	1.2
ANAPC13	Anaphase promoting complex subunit 13	0.02	1.2	0.048	1.2
ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10	0.001	1.2	0.001	1.2

Table 4.4 List of top 20 genes based on Log2 fold change of the high magnesium treatments that were significantly down-regulated by both the high- and low-magnesium treatments relative to the control

Gene Symbol	Gene Title	5 mM vs 1 mM		0.1 mM vs 1 mM	
		p-value	Log2 Fold-Change	*p*-value	Log2 Fold-Change
CLASP1	Cytoplasmic linker associated protein 1	0.007	-1.6	0.032	-1.5
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	0.004	-1.5	0.033	-1.3
AQP1	Aquaporin 1 (Colton blood group)	*P*<0.001	-1.5	0.013	-1.2
C9orf64	Chromosome 9 open reading frame 64	0.002	-1.5	0.039	-1.2
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	0.032	-1.4	0.042	-1.4
C3orf55	Chromosome 3 open reading frame 55	0.03	-1.4	0.033	-1.4
CDH12	Cadherin 12, type 2 (N-cadherin 2)	0.004	-1.4	0.021	-1.3
POSTN	Periostin, osteoblast specific factor	0.019	-1.4	0.02	-1.4
COL1A2	Collagen, type I, alpha 2	*P*<0.001	-1.4	0.003	-1.2
PLEKHA4	Pleckstrin homology domain containing, family A	0.003	-1.3	0.034	-1.2
UGGT2	UDP-glucose glycoprotein glucosyltransferase 2	0.014	-1.3	0.001	-1.6
F8	Coagulation factor VIII, procoagulant component	0.021	-1.3	0.03	-1.3
MME	Membrane metallo-endopeptidase	*P*<0.001	-1.3	0.021	-1.1
TCEAL7	Transcription elongation factor A (SII)-like 7	*P*<0.001	-1.3	0.001	-1.2
HDDC2	HD domain containing 2	*P*<0.001	-1.3	0.01	-1.1
LGR5	Leucine-rich repeat-containing G protein-coupled receptor 5	0.007	-1.3	0.045	-1.2
SOCS2	Suppressor of cytokine signaling 2	0.026	-1.3	0.025	-1.3
THUMPD3	THUMP domain containing 3	0.013	-1.3	0.011	-1.3
SLC16A5	Solute carrier family 16, member 5 (monocarboxylic acid transporter 6)	*P*<0.001	-1.3	0.005	-1.1
FDXACB1	Ferredoxin-fold anticodon binding domain containing 1	0.001	-1.3	0.012	-1.2

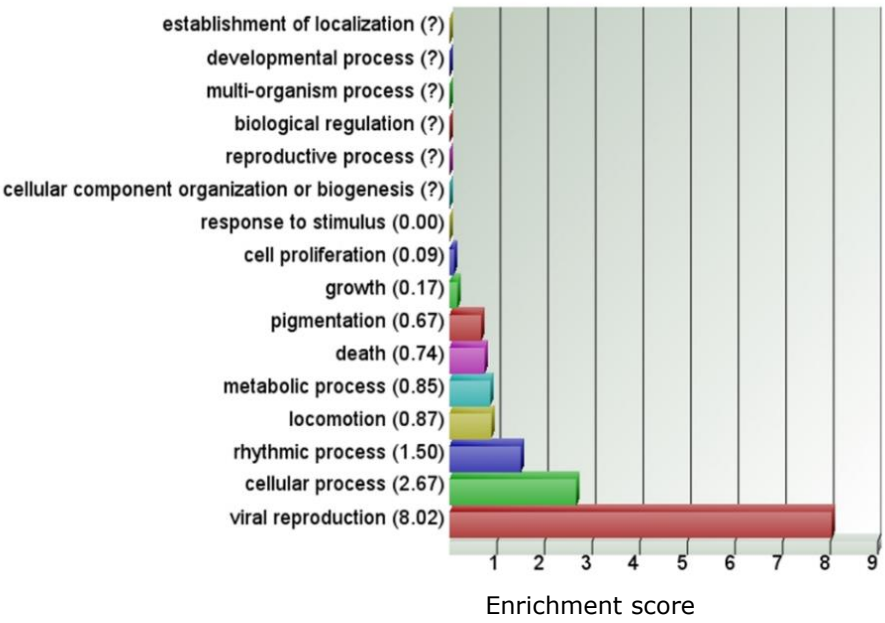
4.6.2 Gene ontology analysis

The results of the microarray analysis were analysed using Gene Ontology (GO) analysis on Partek Genomics Suite software, in order to identify the biological processes, molecular functions and cellular components that were significantly impacted by low or high magnesium concentrations. GO performs enrichment analysis on gene sets by considering the numbers of transcripts associated with a particular cell component or biological process that are differentially expressed in response to a treatment or challenge. For example, given a set of genes that are up-regulated with low magnesium, an enrichment analysis will find which GO terms which are over-represented or under-represented. The main outputs of GO analysis are enrichment scores, with high enrichment indicating that a biological process, cellular component or molecular function is over-represented in a set of differentially expressed genes.

4.6.2.1 Biological processes

Figures 4.5 A and B show the enrichment scores for biological process of HUVECs genes treated with 0.1 mM and 5 mM $MgSO_4$ and were significantly under- or over-expressed compared to the control. For low-magnesium treatment the highest enrichment was noted for viral reproduction and cellular processes, while the high-magnesium treatment increased the enrichment scores for cell proliferation and cell killing. Figures 4.6 A and B show the number of genes regulated in each biological process group, and whether they are up- or down-regulated by the low- or high-magnesium. The figures show that most of the genes regulated by the low- and high-magnesium treatments were involved in viral reproduction, response to stimulus and cell proliferation.

A



B

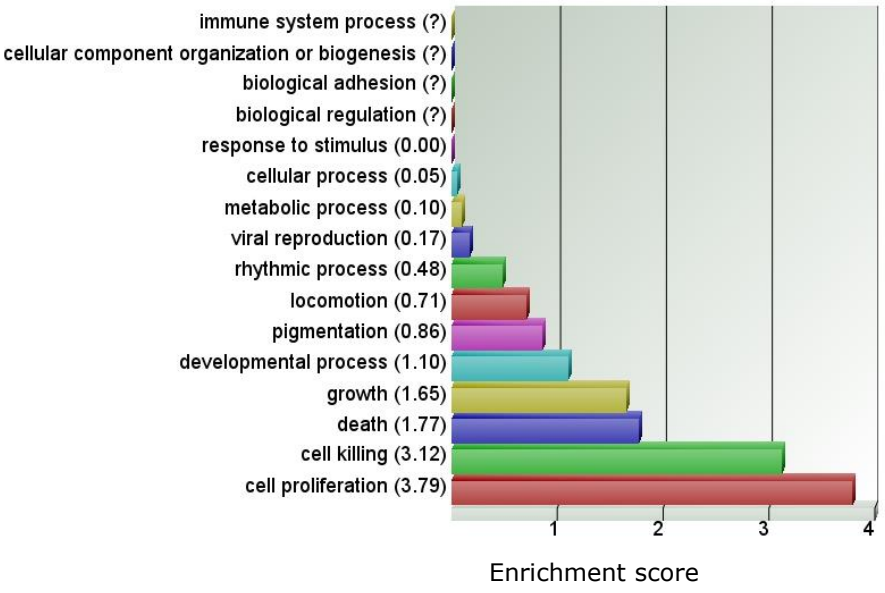
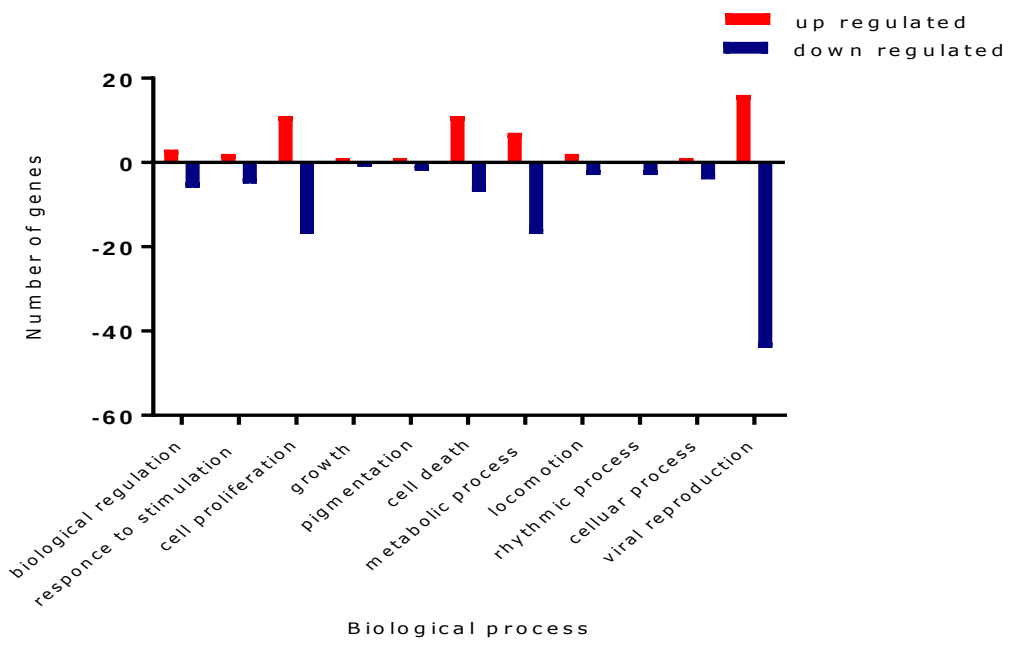


Figure 4.5 GO enrichment analysis of the biological process group using Partek Genomics Suite software. The functional categories are ranked according to their *p* values and Log2 fold change greater than 1. A: Regulated by low-magnesium concentration. B: Regulated by high-magnesium concentration.

A



B

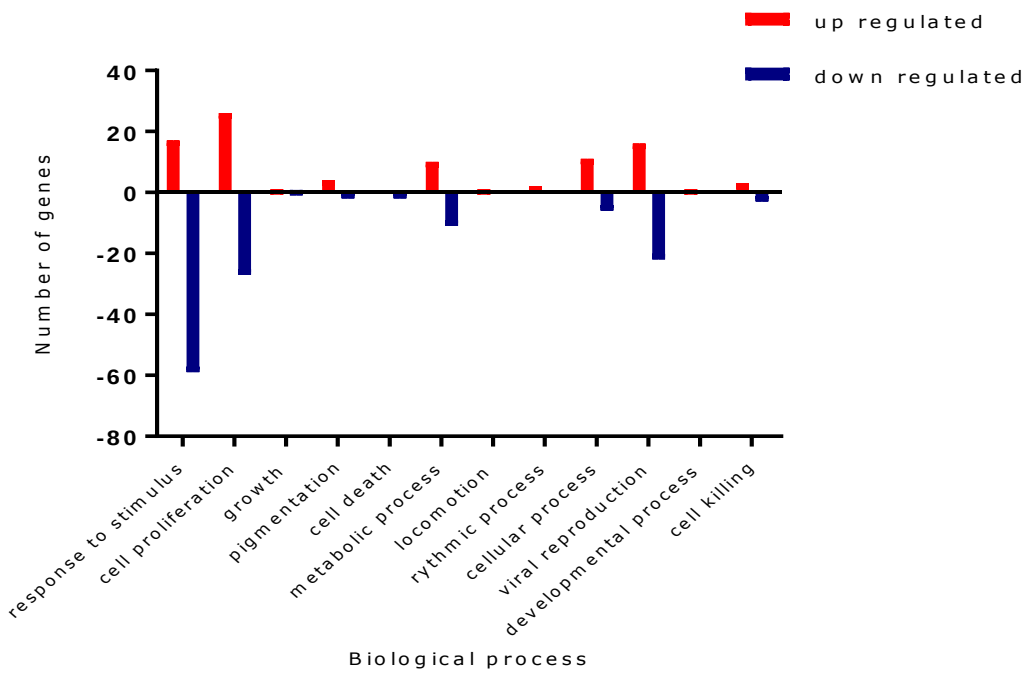


Figure 4.6 Number of genes in the biological process group. $p < 0.05$ and Log2 fold change greater than 1. A: Regulated by low-magnesium concentration. B: Regulated by high-magnesium concentration.

4.6.2.2 Molecular functions

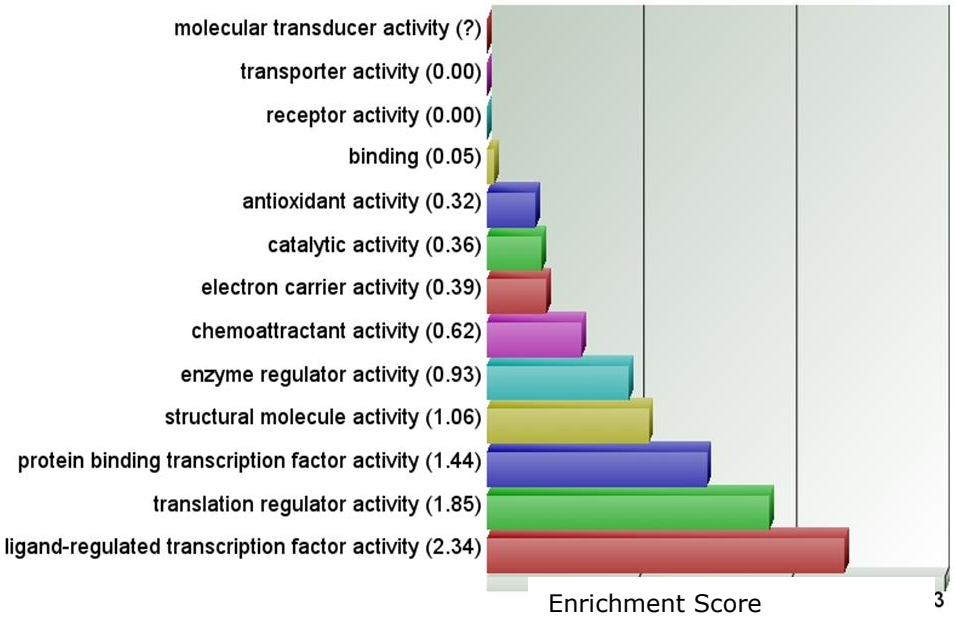
In the GO analysis of molecular function in HUVECs treated with 0.1 mM $MgSO_4$, the highest enrichment score was for ligand regulated transcription factor activity, followed by translation regulator activity and protein binding transcription factor activity. The categories of antioxidant activity, enzyme regulator activity and ligand regulated transcription factor activity were most overrepresented in the molecular function by the high-magnesium treatment (Figures 4.7 A and B).

To present the number of genes significantly expressed in each molecular function group, Figure 4.8 A and B shows the number of genes that were up-regulated or down-regulated by low- and high-magnesium treatment. Binding and the receptor activity groups had the highest group numbers of genes in both up- and down-regulation. In contrast, the genes in the antioxidant activity had the fewest genes in up- and down-regulation.

4.6.2.3 Cellular components

The most overrepresented categories in the cellular component domain were cell junctions for both high- and low-magnesium treatment (Figures 4.9 A and B). In addition, the majority of genes up- and down-regulated by both high- and low-magnesium treatment were involved in the extracellular region (Figures 4.10 A and B).

A



B

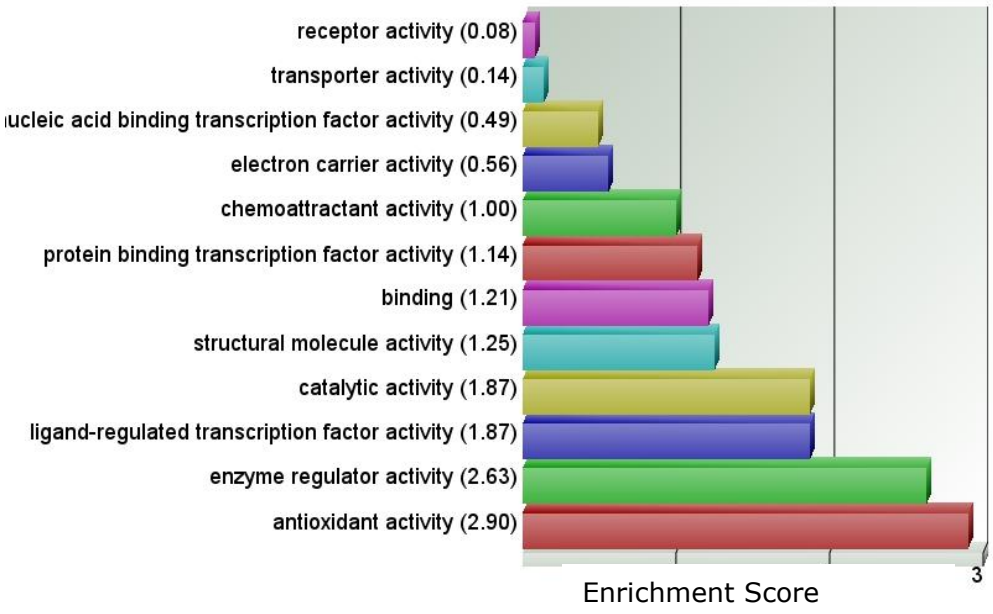
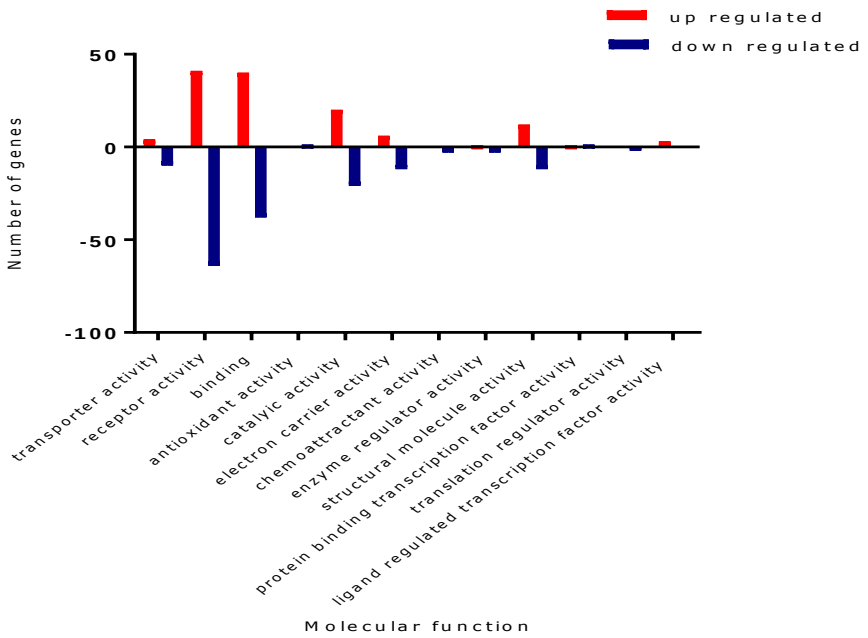


Figure 4.7 GO enrichment analysis of the molecular function group using Partek Genomics Suite software. The functional categories are ranked according to their *p* values and Log2 fold change greater than 1. A: Regulated by low-magnesium concentration. B: Regulated by high-magnesium concentration.

A



B

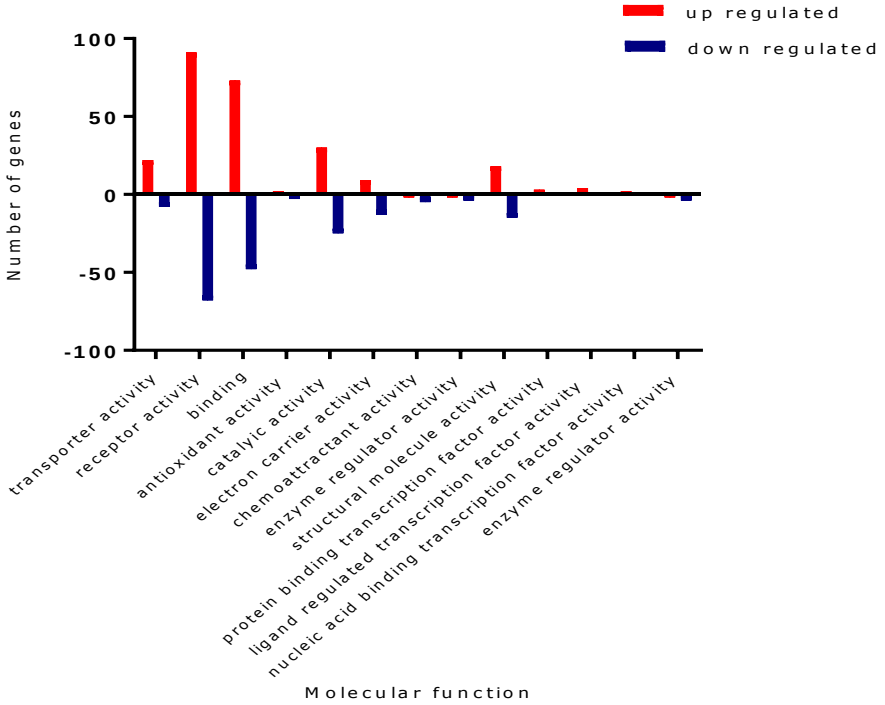
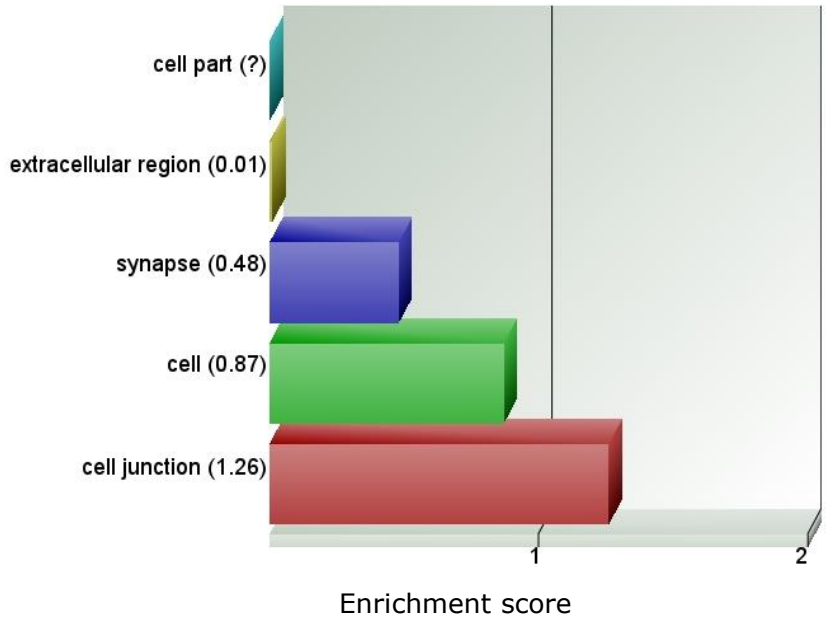


Figure 4.8 Number of genes in molecular function group. $p < 0.05$ and Log2 fold change greater than 1. A: Regulated by low-magnesium concentration. B: Regulated by high-magnesium concentration.

A



B

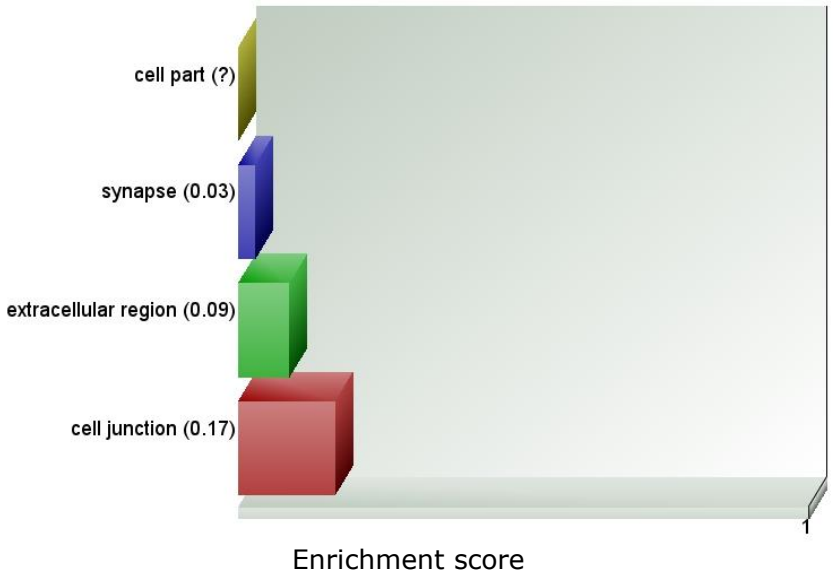
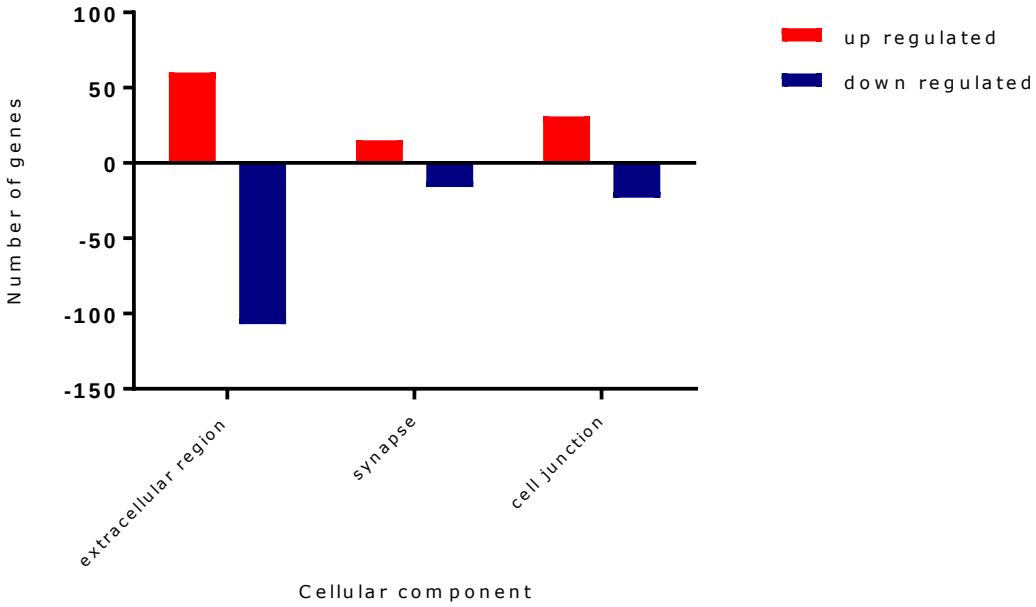


Figure 4.9 GO enrichment analysis of the cellular component group by using Partek Genomics Suite software. The functional categories are ranked according to their *p* values and Log2 fold change greater than 1. A: Regulated by low-magnesium concentration. B: Regulated by high-magnesium concentration.

A



B

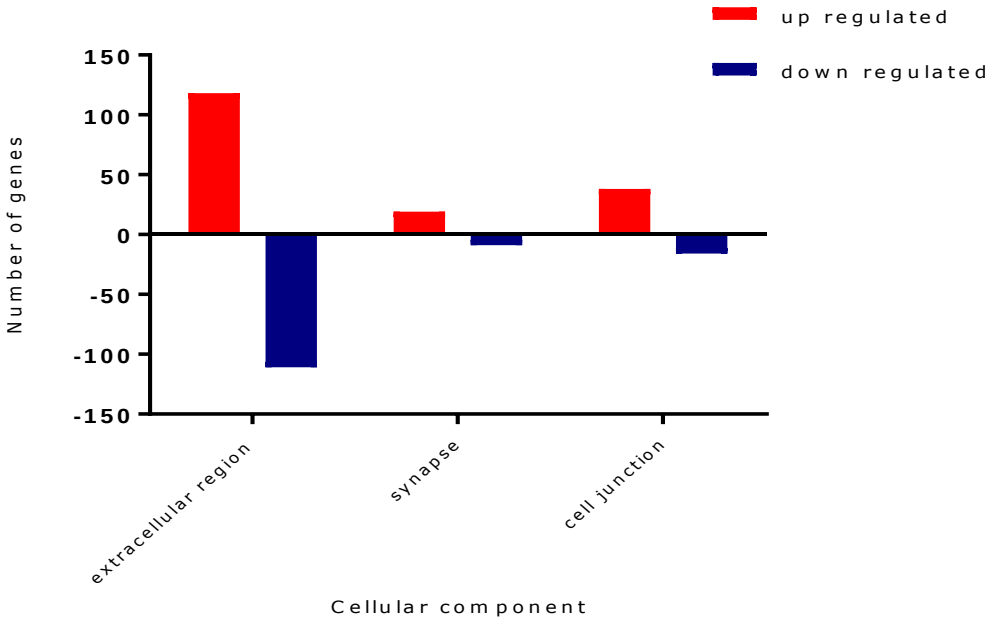


Figure 4.10 Number of genes in the cellular component group. $p < 0.05$ and Log2 fold change greater than 1. A: Regulated by low-magnesium concentration. B: Regulated by high-magnesium concentration.

4.6.3 Effects of Magnesium Concentration on Genes Involved in Inflammation, Mediated by the Chemokine and Cytokine Signalling Pathway

Discussion with Professor Richard Emes (Advanced Data Analysis Centre, University of Nottingham) and preliminary analysis using Ingenuity Pathway Analysis (Qiagen), identified IL-8 and associated inflammatory pathways as a being highly sensitive to varying magnesium concentrations. Given this, and the fact that inflammation plays a key role in endothelial dysfunction, further consideration was given to the GO terms that were most enriched under conditions of varying magnesium. This indicated that responses to stress, including inflammation would be a good focus for further investigation. The effect of magnesium on genes involved in inflammatory pathways was therefore examined using the Gene Ontology (GO) Consortium bioinformatics resource to analyse the microarray data. The results showed that high-magnesium treatment significantly affected 444 genes that respond to stress, 33 of which are involved in inflammation mediated by the chemokine and cytokine signalling pathway (Table 4.5). Ten of these are involved in the interleukin signalling pathway (Table 4.6). In contrast, the low-magnesium treatment significantly altered mRNA expression of 376 genes that respond to stress, 28 of which belonged to the inflammation mediated by chemokine and cytokine signalling pathway (Table 4.7). Five of these genes were a part of the interleukin signalling pathway (Table 4.8).

Table 4.5 Differential expression of 33 transcripts based on fold change annotated to genes that are involved in inflammation mediated by chemokine and cytokine signalling pathway (High magnesium condition)

Gene Symbol	Gene Title	Gene Ontology Biological Process	*p*-value	Log2 Fold-Change	Fold-Change Description
CCR9	Chemokine (C-C motif) receptor 9	Chemotaxis /inferred from electronic annotation / chemotaxis	0.042	1.3	5 mM up vs 1 mM
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	B cell homeostasis / inferred from electronic annotation /	0.004	1.3	5 mM up vs 1 mM
RGS1	Regulator of G-protein signaling 1	Immune response /traceable author statement / signal transduce	0.047	1.2	5 mM up vs 1 mM
RELA	Transcription factor p65	Liver development / inferred from electronic annotation	0.047	1.2	5 mM up vs 1 mM
PDPK1	3-phosphoinositide dependent protein kinase-1	Protein phosphorylation / inferred from electronic annotation /	0.022	1.2	5 mM up vs 1 mM
PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	Superoxide metabolic process /traceable author statement	0.019	1.1	5 mM up vs 1 mM
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	G1/S transition of mitotic cell cycle / inferred from electronic annotation	0.036	1.1	5 mM up vs 1 mM
ARRB1	Arrestin, beta 1	G-protein coupled receptor internalization / inferred from mutant phenotype	0.015	1.1	5 mM up vs 1 mM
RHOG	Rho-related GTP-binding protein, member G (rho G)	Small GTPase mediated signal transduction /inferred from electronic annota	0.035	1.1	5 mM up vs 1 mM
SOS1	Son of sevenless homolog 1	Apoptosis / not recorded signal transduction	0.017	1.1	5 mM up vs 1 mM
GRK6	G protein-coupled receptor kinase 6	Protein phosphorylation / inferred from electronic annotation	0.006	1.1	5 mM up vs 1 mM
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	Blood vessel remodeling inferred from electronic annotation	0.029	1.1	5 mM up vs 1 mM
PTGS1	Prostaglandin-endoperoxide synthase 1	Prostaglandin biosynthetic process / inferred from sequence or structural s	0.017	1.1	5 mM up vs 1 mM
PAK4	P21 protein (Cdc42/Rac)-activated kinase 4	Protein phosphorylation / inferred from electronic annotation	0.019	1.1	5 mM up vs 1 mM
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	Transcription / traceable author statement / signal transducti	0.01	1.1	5 mM up vs 1 mM
RHOC	Rho-related GTP binding protein, member C	Small GTPase mediated signal transduction / inferred from electronic annota	0.018	1.1	5 mM up vs 1 mM

CCL7	Chemokine (C-C motif) ligand 7	Cellular calcium ion homeostasis / traceable author statement	0.042	1.1	5 mM up vs 1 mM
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	Transport / inferred from electronic annotation / ion transport	0.038	1.1	5 mM up vs 1 mM
PTEN	Phosphatase and tensin homolog	Regulation of cyclin-dependent protein kinase activity / traceable author	0.03	1.1	5 mM up vs 1 mM
PLCD1	Phospholipase C, delta 1	Lipid metabolic process /inferred from electronic annotation /	0.03	1.1	5 mM up vs 1 mM
ARAF	Serine/threonine protein kinase A raf	Protein modification process / traceable author statement	*P*<0.001	1.1	5 mM up vs 1 mM
RRAS	Related RAS viral (r-ras) oncogene homolog	Signal transduction /inferred from electronic annotation	0.041	1.1	5 mM up vs 1 mM
CAMK2D	Calcium/calmodulin-dependent protein kinase II delta	G1/S transition of mitotic cell cycle // inferred from electronic annotation	0.038	1.1	5 mM up vs 1 mM
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	Chemotaxis / inferred from electronic annotation /chemotaxis	*P*<0.001	-1.8	5 mM down vs 1 mM
IL8	Interleukin 8	Angiogenesis / traceable author statement / cellular component	0.011	-1.5	5 mM down vs 1 mM
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Chemotaxis /traceable author statement inflammatory response	0.027	-1.2	5 mM down vs 1 mM
NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	Transcription / inferred from electronic annotation	0.033	-1.0	5 mM down vs 1 mM
KRAS	GTPase KRas	Apoptosis /inferred from electronic annotation / signal trans	0.029	-1.1	5 mM down vs 1 mM
RGS14	Regulator of G-protein signaling 14	Mitosis / inferred from electronic annotation / signal transduce	0.001	-1.1	5 mM down vs 1 mM
CCL4	Chemokine (C-C motif) ligand 4	Cellular component movement / traceable author statement	0.008	-1.1	5 mM down vs 1 mM
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	Inflammatory response /inferred from electronic annotation	0.024	-1.1	5 mM down vs 1 mM
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	Cytokinesis / inferred from electronic annotation / protein ph	0.002	-1.1	5 mM down vs 1 mM
CXCL2	Chemokine (C-X-C motif) ligand 2	Chemotaxis / inferred from electronic annotation / chemotaxis	0.045	-1.1	5 mM down vs 1 mM

Table 4.6 Differential expression of 10 transcripts based on fold change annotated to genes that are involved the interleukin signalling pathway (High magnesium condition).

Gene Symbol	Gene Title	Gene Ontology Biological Process	p-value	Log2 Fold-Change	Fold-Change Description
PDPK1	3-phosphoinositide dependent protein kinase-1	Protein phosphorylation /inferred from electronic annotation	0.022	1.2	5 mM up vs 1 mM
NF-κB IB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	Transcription / traceable author statement / signal transducti	0.01	1.1	5 mM up vs 1 mM
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Regulation of cyclin-dependent protein kinase activity / traceable author s	0.006	1.1	5 mM up vs 1 mM
STAT2	Signal transducer and activator of transcription 2	Transcription / inferred from electronic annotation	0.04	1.0	5 mM up vs 1 mM
IL8	Interleukin 8	Angiogenesis / traceable author statement // cellular component	0.011	-1.5	5 mM down vs 1 mM
IL1RL1	Interleukin 1 receptor-like 1	Immune response / non-traceable author statement /signal tran	*P*<0.001	-1.3	5 mM down vs 1 mM
IL31RA	Interleukin 31 receptor A	MAPKKK cascade / non-traceable author statement anti-apoptos	0.028	-1.2	5 mM down vs 1 mM
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	Inflammatory response /inferred from electronic annotation	0.024	-1.2	5 mM down vs 1 mM
IL33	Interleukin 33	Positive regulation of macrophage activation / inferred from direct assay	0.014	-1.2	5 mM down vs 1 mM
IL6R	Interleukin 6 receptor	Hepatic immune response / traceable author statement	0.044	-1.1	5 mM down vs 1 mM

Table 4.7 Differential expression of 28 transcripts based on fold change annotated to genes that are involved in inflammation mediated by chemokine and cytokine signalling pathway (Low magnesium condition)

Gene Symbol	Gene Title	Gene Ontology Biological Process	*p*-value	Log2 Fold-Change	Fold-Change Description
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	B cell homeostasis /inferred from electronic annotation	0.006	1.3	0.1 mM up vs 1 mM
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Prostaglandin biosynthetic process / inferred from sequence or structural	0.008	1.2	0.1 mM up vs 1 mM
PDPK1	3-phosphoinositide dependent protein kinase-1	Protein phosphorylation / inferred from electronic annotation	0.01	1.2	0.1 mM up vs 1 mM
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	Transport / inferred from electronic annotation ion transpor	0.03	1.2	0.1 mM up vs 1 mM
PTEN	Phosphatase and tensin homolog	Regulation of cyclin-dependent protein kinase activity / traceable author s	0.006	1.2	0.1 mM up vs 1 mM
GNG7	Guanine nucleotide binding protein (G protein), gamma 7	Behavioral fear response / inferred from electronic annotation	0.03	1.1	0.1 mM up vs 1 mM
MYLK3	Myosin light chain kinase 3	Protein phosphorylation / inferred from electronic annotation	0.018	1.1	0.1 mM up vs 1 mM
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, M	G1/S transition of mitotic cell cycle / inferred from electronic annotation	0.014	1.1	0.1 mM up vs 1 mM
CAMK2D	Calcium/calmodulin-dependent protein kinase II delta	G1/S transition of mitotic cell cycle // inferred from electronic annotation	0.01	1.1	0.1 mM up vs 1 mM
PAK2	P21 protein (Cdc42/Rac)-activated kinase 2	Protein phosphorylation / inferred from direct assay / protein	0.017	1.1	0.1 mM up vs 1 mM
BRAF	V-raf murine sarcoma viral oncogene homolog B1	MAPKKK cascade /inferred from electronic annotation / protein	0.003	1.1	0.1 mM up vs 1 mM
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	G1/S transition of mitotic cell cycle / inferred from electronic annotation	0.04	1.1	0.1 mM up vs 1 mM
CXCR4	Chemokine (C-X-C motif) receptor 4	Activation of MAPK activity traceable author statement	0.01	-1.3	0.1 mM down vs 1 mM
GRAP	GRB2-related adaptor protein	Ras protein signal transduction /traceable author statement	0.03	-1.2	0.1 mM down vs 1 mM

GNG2	Guanine nucleotide binding protein (G protein), gamma 2	Signal transduction / inferred from electronic annotation	0.01	-1.2	0.1 mM down vs 1 mM
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	Endocytosis / inferred from electronic annotation / apoptosis	0.01	-1.1	0.1 mM down vs 1 mM
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	Leukotriene production involved in inflammatory response / inferred from el	0.04	-1.1	0.1 mM down vs 1 mM
ARRB1	Arrestin, beta 1	G-protein coupled receptor internalization / inferred from mutant phenotype	0.04	-1.1	0.1 mM down vs 1 mM
GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	Transport /non-traceable author statement / vesicle fusion /	0.007	-1.1	0.1 mM down vs 1 mM
RRAS2	Related RAS viral (r-ras) oncogene homolog 2	Signal transduction /inferred from electronic annotation	0.013432	-1.1	0.1 mM down vs 1 mM
CCL4	Chemokine (C-C motif) ligand 4	Cellular component movement /traceable author statement	0.01	-1.1	0.1 mM down vs 1 mM
NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	Transcription / inferred from electronic annotation	0.036	-1.1	0.1 mM down vs 1 mM
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	Apoptosis / inferred from electronic annotation / signal trans	0.005	-1.1	0.1 mM down vs 1 mM
CCRL2	Chemokine (C-C motif) receptor-like 2	Chemotaxis / traceable author statement / signal transduction	0.03	-1.1	0.1 mM down vs 1 mM
IL1B	Interleukin 1, beta	Activation of MAPK activity /inferred from direct assay	0.02	-1.1	0.1 mM down vs 1 mM
PAK4	P21 protein (Cdc42/Rac)-activated kinase 4	Protein phosphorylation /inferred from electronic annotation	0.03	-1.05	0.1 mM down vs 1 mM
RGS14	Regulator of G-protein signaling 14	Mitosis /inferred from electronic annotation / signal transdu	0.004	-1.0	0.1 mM down vs 1 mM
GNG5	Guanine nucleotide binding protein (G protein), gamma 5	Signal transduction /non-traceable author statement / signal	0.03	-1.0	0.1 mM down vs 1 mM

Table 4.8 Differential expression based on fold change of 5 transcripts annotated to genes that are involved the interleukin signalling pathway (Low magnesium condition)

Gene Symbol	Gene Title	Gene Ontology Biological Process	*p*-value	Log2 Fold-Change	Fold-Change Description
RPS6KA2	Ribosomal protein S6 kinase, 90kDa, polypeptide 2	Mitotic metaphase /inferred from electronic annotation	0.01	1.2	0.1 mM up vs 1 mM
PDPK1	3-phosphoinositide dependent protein kinase-1	Protein phosphorylation / inferred from electronic annotation	0.01	1.2	0.1 mM up vs 1 mM
BRAF	V-raf murine sarcoma viral oncogene homolog B1	MAPKKK cascade /inferred from electronic annotation / protein	0.003	1.1	0.1 mM up vs 1 mM
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	Apoptosis / inferred from electronic annotation / signal trans	0.005	-1.1	0.1 mM down vs 1 mM
IL4	Interleukin 4	Chemotaxis / traceable author statement / immune response	0.04	-1.1	0.1 mM down vs 1 mM

Inflammatory pathways therefore appear to be responsive to low and high magnesium concentrations. A more detailed investigation of these pathways and processes will presented in Chapter 5.

4.6.4 Candidate Gene Approach: Effects of Different Concentrations of Magnesium on the Expression of Key Genes Involved in Endothelial Cell Functions

To further evaluate the effects of magnesium on mRNA and protein expression in HUVECs we followed a candidate gene approach to consider expression of a magnesium transporter and genes and proteins associated with production of nitric oxide and endothelin. We hypothesised that all of these processes would be sensitive to magnesium concentration.

4.6.4.1 Effect of Different Concentrations of Magnesium on the mRNA and Protein Expression of the Transient Receptor Potential Channel-7 (TRPM-7)

TRPM-7 is an ion channel that regulates Mg^{2+} homeostasis (Paravicini et al., 2012). This study examined the influence of $MgSO_4$ on the expression of TRPM-7 mRNA in HUVECs. Figure 4.11 shows that a low $MgSO_4$ concentration modulated TRPM-7 mRNA in HUVECs, culturing the cells in medium containing 0.1 mM $MgSO_4$ resulted in a significant increase in TRPM-7 mRNA at 24 (P= 0.003, Dunnett test) and 72 hours (P= 0.027, Dunnett test), compared to the cells that were cultured in medium containing 1 mM $MgSO_4$. TRPM-7 is upregulated in the 0.1 mM medium to accommodate the need for increased magnesium uptake. No significant difference in TRPM-7 mRNA was observed with 5 mM $MgSO_4$.

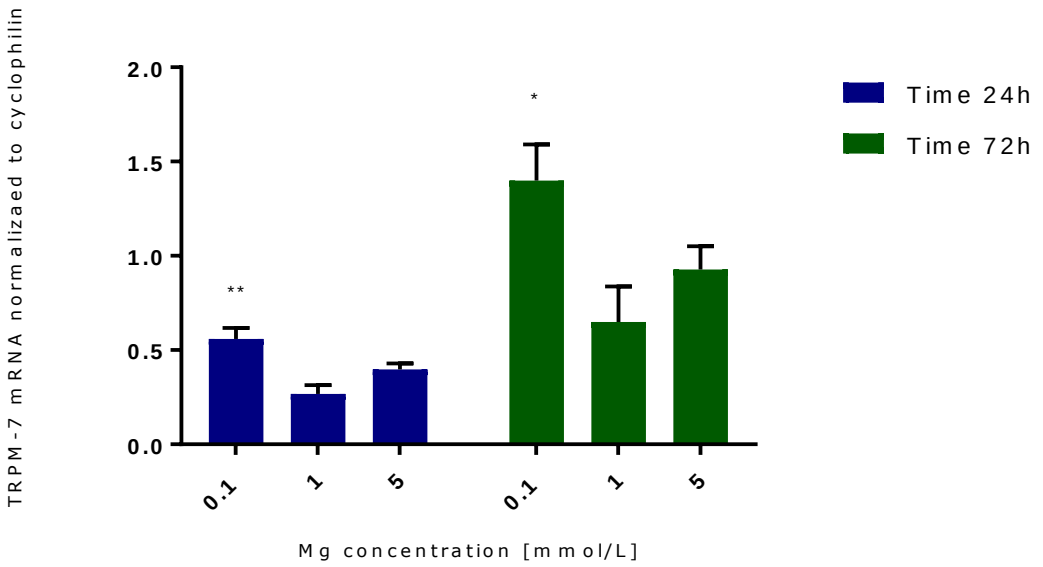
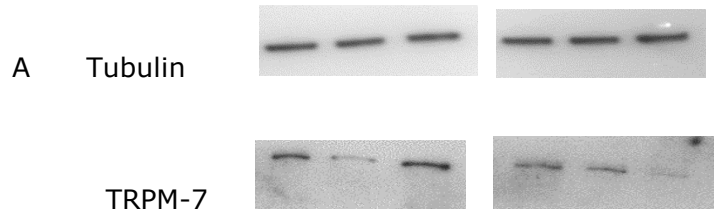


Figure 4.11 Concentration-dependent and time-dependent effects of $MgSO_4$ in the culture medium on HUVECs expression of TRPM-7 mRNA. HUVECs culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for 24 h and 72 h. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean±SEM for n = 4. Two-way ANOVA showed no significant interaction of magnesium × time (P= 0.117). There was a significant effect of magnesium concentration (P = 0.002) and time (P<0.001). * indicates P<0.05 and ** indicates P<0.01, compared to physiological 1 mM $MgSO_4$ in the same time point (Dunnett's test).

The results shown in Figure 4.12 show the effect of different magnesium concentrations on the protein concentration of TRPM-7 in HUVECs using Western blot analysis with alpha tubulin used as an internal control. The band was visible at ~250 KDa, the expected size for the protein, and was the only band on the gel. TRPM-7 protein expression was significantly increased (*P*= 0.002, Dunnett test) when HUVECs were cultured in a medium with a low magnesium concentration for 72 hours. On other hand, a high magnesium concentration decreased TRPM-7 protein concentration after 72 hours (*P*= 0.013, Dunnett test) compared to the control. No differences in expression were noted after 24 hours culture.



B

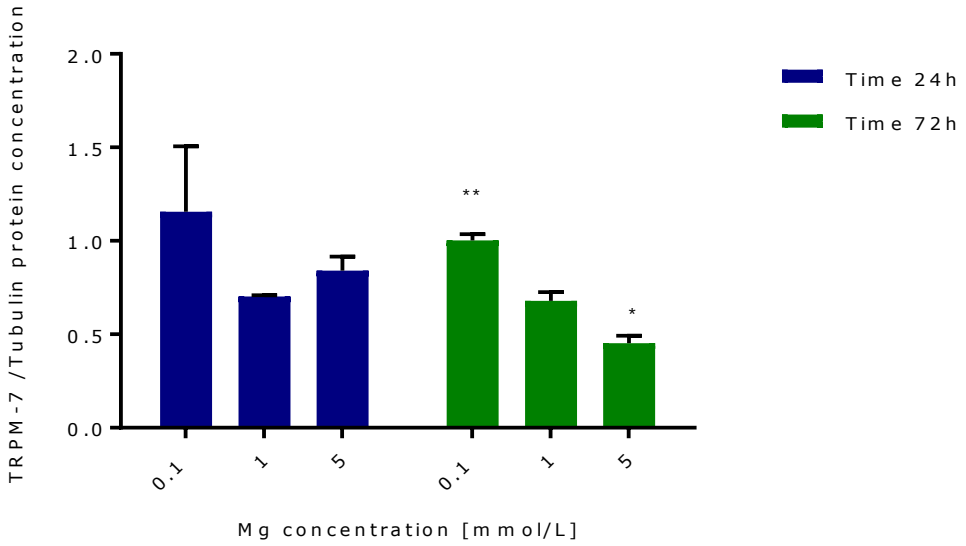


Figure 4.12 Western blot analysis of the effect of $MgSO_4$ concentration on TRPM-7 protein levels in HUVECs cultures. Lysed cells were prepared for western blot analysis using anti-TRPM-7 antibodies, as indicated in the Materials and Methods section of this work. The same blot was also analysed with anti-tubulin antibodies. A: Representative bands of TRPM-7 and tubulin. B: A marked increase in TRPM-7 protein expression at low magnesium concentrations at 72 h and a significant decrease at high magnesium concentrations. Data are shown as mean±SEM for n = 3. Two-way ANOVA showed no significant interaction of magnesium × time (*P*= 0.48). There was a significant effect of magnesium concentration (*P* = 0.025). * indicates *P*<0.05 and ** indicates *P*<0.01, compared to physiological 1 mM $MgSO_4$ in the same time point (Dunnett's test).

4.6.4.2 Effect of Different Concentrations of Magnesium on the Gene Expression of Endothelin-1 (ET-1)

Endothelial cells control vasoconstricion through the release of ET-1 which impacts on smooth muscle cells (Laurant and Berthelot, 1996). The expression of ET-1 mRNA was examined by culturing HUVECs for 24 and 72 hours in media containing 0.1, 1 and 5 mM $MgSO_4$, and then conducting qRT-PCR. Figure 4.13 shows that ET-1 mRNA in HUVECs was not significantly affected by different magnesium concentration after 24 or 72 hours in culture.

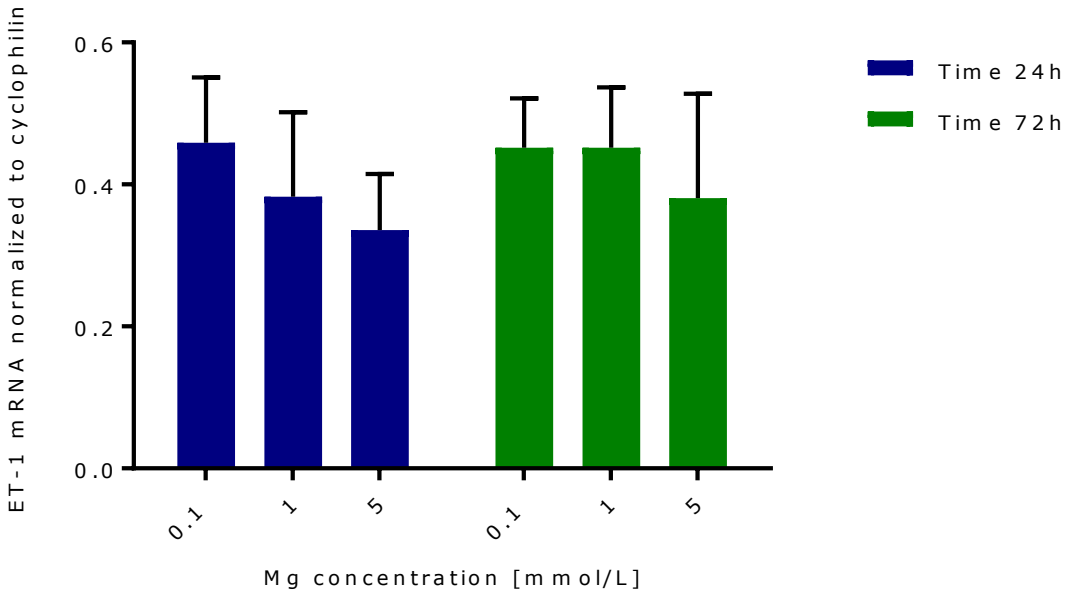


Figure 4.13 Concentration-dependent and time-dependent effects of $MgSO_4$ medium on HUVECs expression of ET-1 mRNA. HUVECs was cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for 24 h and 72 h. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean±SEM for n = 4. Two-way ANOVA showed no significant interaction of magnesium × time (P= 0.91). There was no significant effect of magnesium concentration (P= 0.59) or time (P=0.63).

4.6.4.3 Effect of Different Concentrations of Magnesium on Nitric Oxide Synthase (eNOS) expression and Nitric Oxide Production

eNOS is an important enzyme released from endothelial cells which regulates vascular vasodilation through the production of NO gas (Kietadisorn et al., 2012). The effects of different concentrations of $MgSO_4$ in the culture medium on eNOS mRNA in HUVECs cells are shown in Figure 4.14. HUVECs were cultured for 24 and 72 hours in media containing 0.1, 1 and 5 mM $MgSO_4$ and then were subjected to qRT-PCR. Low and high $MgSO_4$ did not have significant effects on the expression of eNOS mRNA at 24 and 72 hours relative to the physiological concentration of magnesium. Additionally, incubation at different magnesium concentrations for 72 hours did not significantly affect the expression of eNOS in HUVECs stimulated with LPS (0.5 µg/ml) for 4 hours (Figure 4.15).

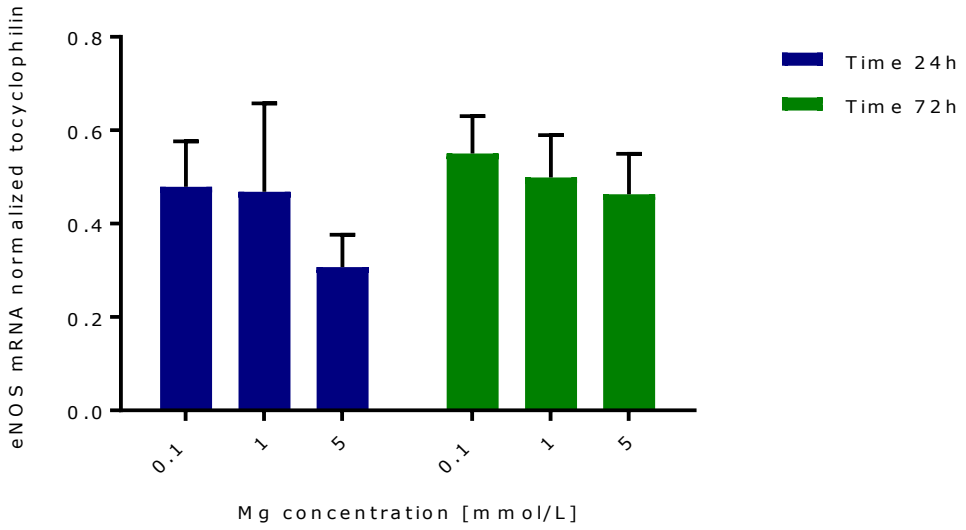


Figure 4.14 Concentration-dependent and time-dependent effects of $MgSO_4$ culture medium on HUVECs expression of eNOS mRNA. HUVECs was cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for 24 h and 72 h. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean±SEM for n = 4. Two-way ANOVA showed no significant interaction of magnesium × time (P=0.84). There was no significant effect of magnesium concentration (P= 0.45) or time (P= 0.32).

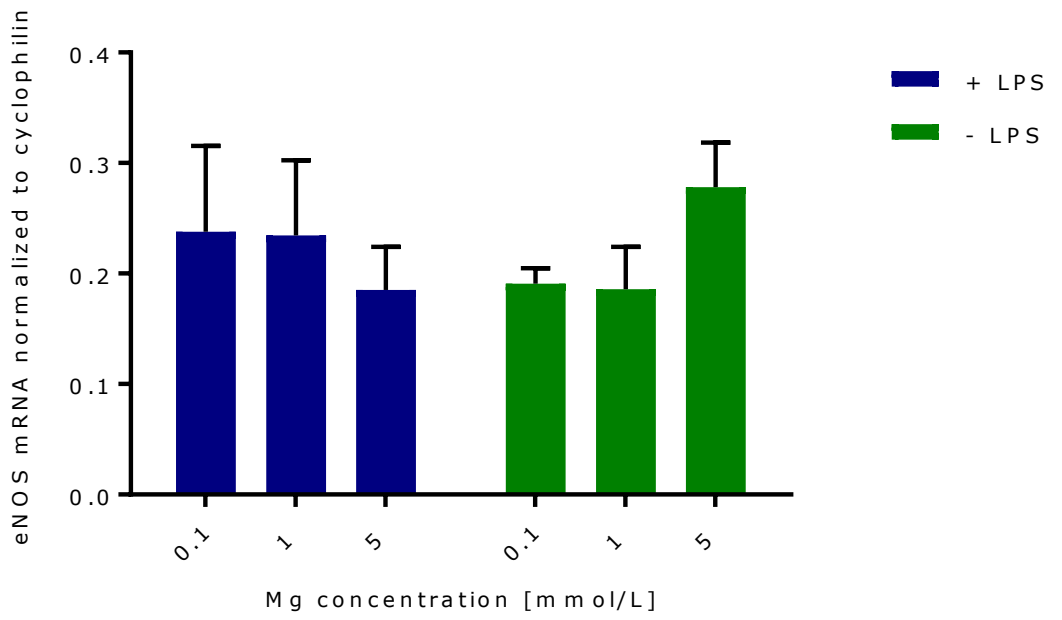


Figure 4.15 Effect of different concentrations of magnesium on eNOS mRNA expression. After 3 days in culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM), confluent HUVECs were stimulated with or without LPS (0.5 μg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean+SEM for n = 4. Two-way ANOVA showed no significant interaction of magnesium × LPS (P=1). There was no significant effect of magnesium concentration (P=1) or LPS (P= 0.33).

Western blot analyses were performed, revealing a clear band of ~140 KDa (the expected size of the protein and the only band on the blot). The results showed that eNOS protein concentration did not significantly differ between low and high magnesium concentrations relative to the physiological concentration of magnesium (Figure 4.16). In order to assess the nitric oxide pathway at a more functional level, nitrite which is the major product of NO in the cell, was measured in the supernatant of culture-stimulated HUVECs using the Sievers Nitric Oxide Analyzer (NOA 280i). As shown in Figure 4.17, magnesium treatment did not significantly alter the production of nitric oxide in stimulated HUVECs relative to the control.

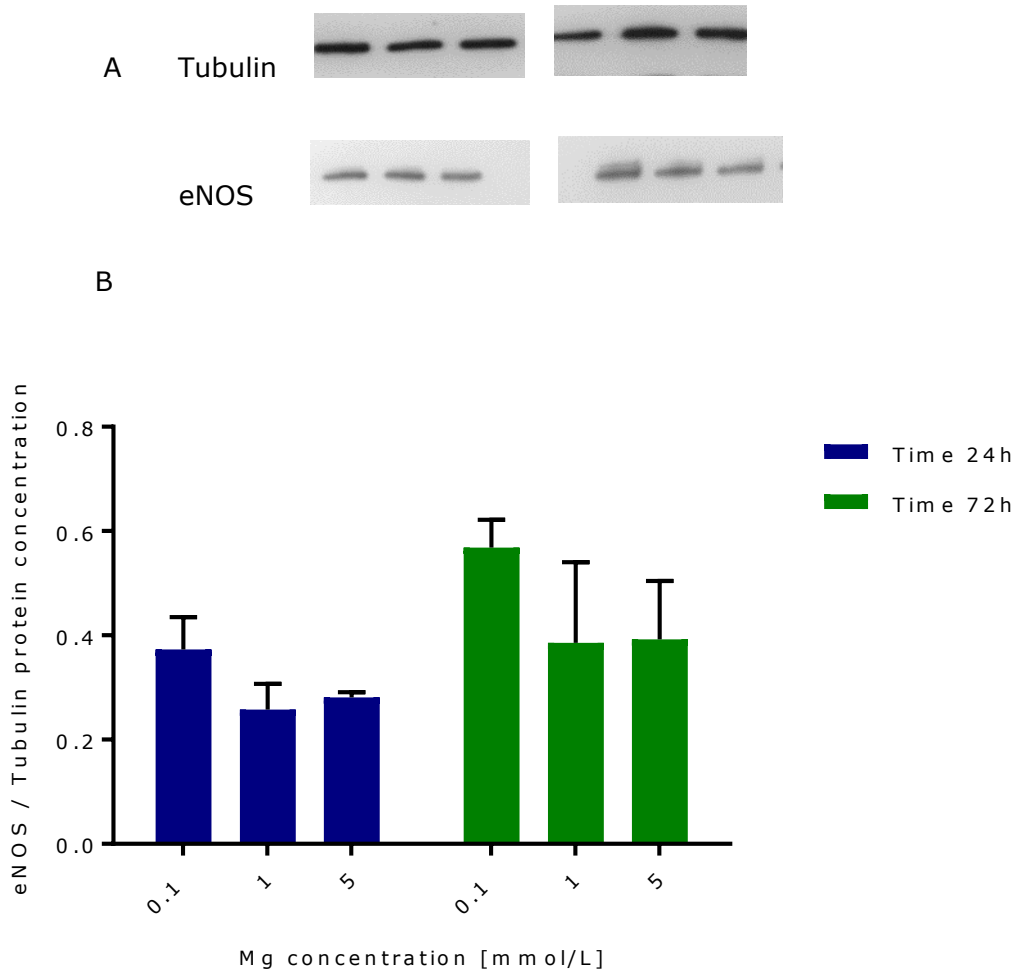


Figure 4.16 Western blot analysis of the effect of $MgSO_4$ concentration on eNOS protein levels in cultured HUVECs. Lysed cells were prepared for western blot analysis using anti-eNOS antibodies, as indicated in the Materials and Methods section of this work. The same blot was reported with anti-tubulin antibodies. A: Representative bands for eNOS and tubulin. B: Insignificant increase in eNOS protein expression at a low magnesium concentration at 72 h. Data are shown as mean±SEM for n = 3. Two-way ANOVA showed no significant interaction of magnesium × time (P=0.88). There was no significant effect of magnesium concentration (P= 0.21) or time (P= 0.065).

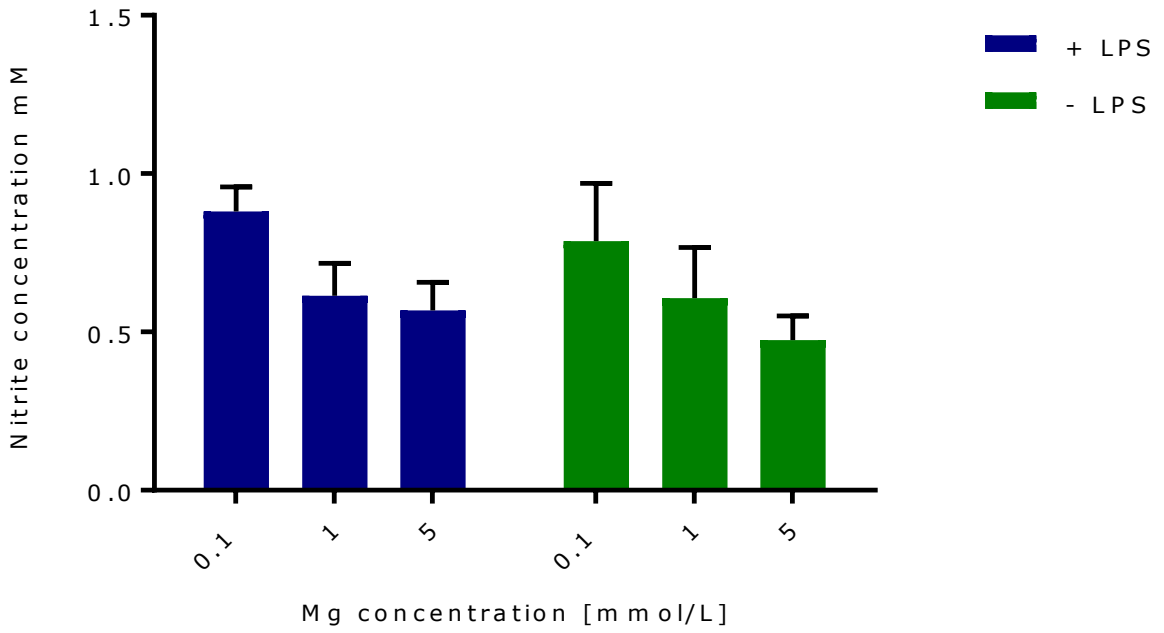


Figure 4.17 Effect of different concentrations of magnesium on nitric oxide production. After 3 days in culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM), confluent HUVECs were stimulated with LPS (0.5 µg/ml) for 4 hours. Supernatants were collected and measured for NO production using the Sievers Nitric Oxide Analyzer (NOA 280i). Data are shown as mean±SEM for n = 4. Two-way ANOVA showed no significant interaction of magnesium × presence of LPS (P= 0.91). There was no significant effect of magnesium concentration (P= 0.89) or presence of LPS (P=0.15).

4.7 Discussion

4.7.1 Effect of Magnesium Concentration on HUVECs' Gene Expression and Gene Ontology Processes

Magnesium is one of the most important cations in human cells because it is critical for many biochemical reactions. Magnesium has been reported to regulate endothelial function, and low magnesium has been associated with endothelial dysfunction (Maier, 2012). Mg^{2+} is also necessary for the regulation of genetic stability (De Baaij et al., 2015). To examine how magnesium can change the expression of mRNAs in HUVECs, this study employed microarray analysis. Microarray technology is a system that can analyse the expression of thousands of genes simultaneously (Huang and Zhang, 2012). This makes it a useful tool for generating further hypotheses for study, and for examining the effects of treatments on broader cellular processes and gene networks. The data from the microarray analysis were gathered using different software packages (Affymetrix MAS 5.0 software, Partek Genomics Suite software and Gene Ontology (GO)) to identify and classify the genes that were significantly altered in HUVECs which were cultured with either a high or a low magnesium concentration. A 3D PCA plot confirmed the high quality of the results by clustering similar gene patterns that behaved analogously to identical mechanisms (Zhang et al., 2009). The PCA plot determined that each treatment group's sample had a separate cluster. This was important as it showed that within-group variation was low and that there were clear differences in response to the magnesium treatments regarding altered gene expression.

Findings presented in the Venn diagram showed that 12.3% of the genes on the microarray were responsive to magnesium treatment, with 6.5% changing due to high magnesium treatment and the remaining 5.8% changing due to low magnesium treatment. In addition, 54% of the genes that were affected by high magnesium were upregulated, and 46% were downregulated, while 47% of the low magnesium group genes were upregulated, and 52% were downregulated. These data suggest that magnesium has a powerful effect on the HUVECs transcriptome. In comparison, Nicholson et al. (2008) found that around 6% of the genome was differentially expressed when HUVECs were treated with three

polyphenolic compounds, ferulic acid, quercetin and resveratrol. In contrast, Chen et al. (2007) found that only 1.5% of the HUVECs genome was impacted by E coli derived LPS. Bal et al. (2012) stimulated HUVECs with different interleukins and found that stimulation of HUVECs with IL-β changed 1% of the gene profile, while IL3 affected 1.4% and IL6 changed around 1.5% of the HUVECs genome.

To our knowledge, this study is the first to present the microarray gene expression profile of the effect of magnesium on HUVECs. Importantly, the study found that magnesium can alter a high percentage of HUVECs genes involved in essential physiological pathways and highlighted the critical role of magnesium in inflammation, which is the focus of the next chapter.

Heat maps help visualize array data via colour, by placing large data tables in cluster rows and columns to find gene expression patterns (Zhao et al., 2014). The heat map for the 615 transcripts that were commonly differentially expressed in both high and low magnesium treatment, showed that the physiological magnesium concentration group matrix clustered differently between the high and low groups. By analysing these genes via gene ontology, it was found that 13% of these genes were related to the cytoskeleton and 17% and 52% were involved in anion and protein binding, respectively. Magnesium plays a role in cytoskeleton formation during the mitotic spindle and cytokinesis (Wolf and Trapani, 2008), and the present data suggest that deviation away from optimal concentration (either deficiency or excess) can disrupt this process. Moreover, any variation in the magnesium concentration can affect the other cell ions, such as Ca^{2+}, Na^{+} and K^{+} (Wolf and Trapani, 2008, Saris et al., 2000). The efflux of Ca^{2+} can be influenced by the concentration of Mg^{2+} on the membrane binding sites because the magnesium ion is a Ca^{2+} antagonist. In addition, K^{+} transport is controlled by Mg^{2+} through the Na^{+}-K^{+} ATPase pump. Further, extracellular magnesium can impact the ionized and protein bonds (Noronha and Matuschak, 2002). This study did not seek to determine why both low- and high-magnesium exert very similar effects on these processes, but the observation is noteworthy. To best to our knowledge no other study has demonstrated that the low and

high concentration of magnesium have similar effects on a large number (581) of HUVECs genes.

To identify the functional processes that were affected by magnesium treatment, Partek Genomics Suite software was used to analyse the microarray data and indicated that the most affected biological process were viral reproduction process, (in which a virus is a participant); the cellular process (the process that is carried out at the cellular level), cell proliferation (multiplication or reproduction of cells) and cell killing (any process in an organism that results in the killing of its own cells) all the process definition from The QuickGO browser (http://www.ebi.ac.uk/QuickGO). As in Chapter 3 it was noted that HUVECs had lower proliferation and reduced viability, these processes may be of some significance.

The results of this study support those of others that have noted magnesium's ability to regulate the cell proliferation by controlling many steps in the cell cycle, whereas low magnesium can increase the expression of cell cycle inhibitors p27 and p21 (Wolf and Trapani, 2008, Wolf and Cittadini, 1999). Decreases in magnesium concentration lead to cell death due to increases in intracellular Ca^{2+} (Noronha and Matuschak, 2002), which result in DNA degradation (Wolf and Cittadini, 1999).

In terms of molecular function, the most overrepresented categories were ligand regulated transcription factor activity (a DNA binding transcription factor activity that is directly regulated by binding of a ligand to the protein with this activity), translation regulator activity (any molecular function involved in the initiation, activation, perpetuation, repression or termination of polypeptide synthesis at the ribosome), antioxidant activity (inhibition of the reactions brought about by dioxygen (O_2) or peroxides) and enzyme regulator activity (binds to and modulates the activity of an enzyme). The most altered genes in the cellular component domain were involved in cell junctions (a cellular component that forms a specialized region of connection between two or more cells or between a cell and the extracellular matrix) all the process definition from The QuickGO browser (http://www.ebi.ac.uk/QuickGO). These observations are in agreement with

previous studies that have demonstrated that many cellular processes, such as DNA transcription and protein synthesis, require magnesium (De Baaij et al., 2012, Noronha and Matuschak, 2002, Wolf and Cittadini, 1999). Furthermore, magnesium plays a biological role as an important ligand in the cell (Wolf and Trapani, 2008), and low magnesium can impact whether transcription factors bind to the DNA (Noronha and Matuschak, 2002). Mg is essential for several enzyme activities due to its roles as a cofactor (De Baaij et al., 2012, Saris et al., 2000, Wolf and Cittadini, 1999) and an allosteric modulator, which is related to its double-positive charge (Wolf and Trapani, 2008). Wolf et al. (2008b) found that HUVECs cultured in 0.1 mM $MgSO_4$ were associated with increasing oxidant stress as measured by dichlorofluorescein fluorescence. The range of cellular components and molecular functions impacted by variation in magnesium was large. There is considerable evidence here that the magnesium environment in culture will determine how cells respond to further environmental challenges, such as inflammation.

It was not in the scope of this thesis to investigate the full range of processes identified as magnesium-dependent by the microarray. We instead focused upon inflammation as the main pathway of interest, as inflammatory influences on endothelial cell function are the main driver of atherosclerosis *in vivo*. Data from the array (see section 4.6.3) confirmed that inflammatory processes were responsive to variation in magnesium in culture.

4.7.2 Effect of Magnesium Concentration on Genes Involved in Inflammation Mediated by Chemokine and Cytokine Signalling Pathway

Endothelial dysfunction is a major atherosclerosis risk factor (Zhang et al., 2010). Many studies have noted that inflammation promotes formation of atherosclerotic lesions (Apostolakis et al., 2006, Charo and Taubman, 2004). In addition, other studies have found that magnesium plays a role in the regulation of inflammation, and low magnesium is associated with an increase in the inflammatory response (Sugimoto et al., 2012, Mazur et al., 2007, Bernardini et al., 2005). Chemokines are important for inflammation

activation due to their role in regulating immune cells (Murdoch and Finn, 2000). To investigate the effect of different magnesium concentrations on inflammation GO analysis considered inflammatory pathways in more detail using the microarray data.

The array results demonstrated that mRNA expression for many of the interleukin genes, such as IL8, IL5, IL33, IL6R, IL1RL1 and IL31RA, and chemokine genes, such as CXCL6 (GCP-2), CXCL1 (GROα) and CXCL2 (GROβ), was downregulated by high magnesium treatment. However, with low magnesium treatment, IL4 was the only interleukin that was downregulated, while CCL4 (MIP-1β), CXCR4 and CCRL2 were the chemokines that were downregulated.

In the endothelial cell, IL8 and GROα were indicated as pro-inflammation chemokines, which play a role in the transport of monocytes to the sub-endothelial cells and thus initiate atherosclerotic plaque formation (Papadopoulou et al., 2008). The results from the microarray confirmed that mRNA expression of the IL8 and GROα cytokines decreased in the presence of a high magnesium concentration. These findings are the basis of further investigations, which are detailed in the next chapter on how magnesium alters these chemokines and by which pathway it does so. The microarray data and associated GO analysis has clearly indicated that magnesium is a potent modulator of gene expression in HUVECs and that inflammatory processes are among those which are responsive to variation in magnesium concentration.

4.7.3 Effects of Different Concentrations of Magnesium on the Expression of Key Genes Involved in Endothelial Cell Functions

TRPM-7 cation channels, which are expressed in endothelial cells, transport some cations, including magnesium and calcium. TRPM-7 is required for many cell functions, especially proliferation due to its role in controlling magnesium homeostasis, which is important for the influx of magnesium (Inoue and Xiong, 2009). TRPM-7 is also a magnesium-inhibiting channel, so excess intracellular Mg^{2+} and Mg^{2+}-ATP produced by a negative feedback voltage-independent mechanism can inhibit TRPM-7 expression through protein α-kinase domains to decrease the level of Mg^{2+} in the cell (Touyz, 2008, Sontia and Touyz, 2007).

The findings of the present study were consistent with this existing view of how TRPM-7 responds to magnesium, as we found no significant decrease in the protein concentration of TRPM-7 after HUVECs were cultured in high magnesium concentrations for 72 hours. Baldoli et al. (2013) noted that the decline in TRPM-7 expression in the presence of high magnesium concentrations helps protect the cell from imbalanced ion concentrations. On the other hand, low magnesium concentrations significantly increased the mRNA expression of TRPM-7 in HUVECs and the protein expression of TRPM-7. This is consistent with the results of (Baldoli et al., 2013), who found that a medium with a low magnesium concentration (0.1 mM $MgSO_4$) upregulated the gene expression of TRPM-7 in HUVECs compared to a 1 mM $MgSO_4$ medium. This is proposed to occur in order to provide cells with sufficient magnesium and maintain cellular functions. Using qPCR, an animal study found that gene expression of TRPM-7 in endothelial cells from mice selected for low erythrocyte magnesium was noticeably increased in vascular tissue compared to cells from mice selected for high erythrocyte magnesium (Paravicini et al., 2009).

Magnesium is a fundamental cation involved in many cellular reactions. In addition, abnormal magnesium status is related to the pathogenesis of many cardiovascular diseases, especially thrombosis, hyperlipidaemia and hypertension (Champagne, 2008). Through its effects on endothelial homeostasis, magnesium can alter the levels of many vasodilators and vasoconstrictors, which have essential roles in the regulation of vascular circulation and tone (Maier et al., 2004b). For example, nitric oxide is produced by nitric oxide synthase, and serves as a vasodilator, whereas endothelin-1 acts as a vasoconstrictor. During endothelial dysfunction, reduction in vasodilation increases the number of platelets and adhesion molecules, thrombogenicity and inflammation, which can, in combination, lead to cardiovascular events (Shechter and Sherer, 2003).

Endothelin-1 is a protein produced by endothelial cells, that affects smooth muscles by promoting blood flow and controlling blood vessel homeostasis by inducing contraction (Laurant and Berthelot, 1996). Excess production of this protein is associated with a decrease in the amount of nitric oxide, increased hypertension and increased oxidative

stress due to its constrictive effect on vessels (Schiffrin, 2001). The current study examined the mRNA concentration of endothelin-1 in HUVECs cultured at different magnesium concentrations. The results showed no significant variations between different concentrations. Conversely, another study indicated that DOCA-salt hypertensive rats fed 8g/kg diet magnesium supplements for 8 weeks showed a reduction in the gene expression of ET-1 in the aorta and heart as well as decreased blood pressure compared to controls, which were fed 1.5 g/kg diet magnesium for the same period (Berthon et al., 2003). Serebruany et al. (1997) found that normal volunteers given 1g/day of magnesium for two weeks showed significantly decreased levels of plasma ET-1 compared levels before the treatment. Champagne (2008) reported that magnesium deficiency can cause hypertension due to its impact on vasoconstrictors and vasorelaxants. Patients with hypertension, that were given 548 mg magnesium supplements each day for 4 weeks, showed a marked decrease in blood pressure (Itoh et al., 1997). The result of this study does not align with these other studies, possibly because of important differences between in *vitro* and in *vivo* situations where other factors are likely to be regulating expression independently or co-dependently with magnesium (endocrine responses that are absent in culture for example). Moreover, presumably the doses given in these studies (1g/d in humans and 8 g/kg diet in rats) were beyond normal physiological doses. The reference nutrient intake (UK RNI) is 300 mg/d for men, 270 mg/d for women (Vormann, 2003). The AIN93G formulation for rodents is only 0.25-0.5 g/d (Reeves, 1997).

The results of the present study suggest that varying the magnesium concentrations did not significantly change eNOS mRNA, protein concentration or nitric oxide production in HUVECs. This result conflicts with other studies, which found that culturing HUVECs in 5 mM magnesium for 3 days led to increased NOS activity, compared to those cultured in 1 mM magnesium (Maier et al., 2004a). Paravicini et al. (2009) showed that endothelial cell eNOS protein expression and plasma nitrate levels were low in mice selected for low erythrocyte magnesium and were associated with an increase in reactive oxygen species.

On the other hand, other studies have shown that culturing microvascular endothelial cells in a medium with a low magnesium concentration (0.1 mM) for 3 days, increases eNOS activity compared to the control (1 mM concentration) and culturing those cells in a medium with a high magnesium concentration (5 mM) decreases eNOS activity (Bernardini et al., 2005). Howard et al. (1995) found that high magnesium concentrations (10 mM $MgCl_2$) significantly decreased nitric oxide release and NOS activity in HUVECs. Furthermore, stimulating human lung microvascular endothelial cells with 5 ng/ml IL-1B for 24 hours increased nitric oxide production due to inflammation, which can result in apoptosis and DNA damage due to the production of peroxynitrite (Lowry et al., 2013). Both oxidized LDL and oxidative stress increase in the presence of nitric oxide and peroxynitrite, which in turn increase in the presence of atherosclerosis and diabetes (Liu and Huang, 2008). Thomas et al. (2002) found that stimulating aortic endothelial cells with H_2O_2 increases eNOS activity. Maier (2012) clarified that a high concentration of nitric oxide can be harmful to elements of cells. An animal study found that feeding rats a magnesium deficient diet (2 mM /kg diet) for three weeks increased the nitric oxide production relative to the sufficient group 25 mM /kg diet (Mak et al., 1996). The current study results were different to Maier et al. and Howard et al. Which were the only previous studies on HUVECs. Explanations for this discrepancy may be that the other studies measured the total NOS activity (eNOS and iNOS), whilst our study focused on eNOS. Furthermore, the Maier study did not show a statistically significant effect of high magnesium. The Howard et al study utilized a high magnesium concentration that was 10-fold higher than physiological.

4.8 Limitations

This study has generated a wealth of data, providing the basis of further studies to examine the impact of magnesium deficiency or excess on different pathways. There is no scope to examine all of the research questions that arise within the lifetime and funding limits of this study. Due to a limited budget and the high price of the microarray analysis, this study was only able to examine cells cultured in different magnesium concentrations for 24 hours. More informative data may be obtained by further examining the cells for 72

hours, as well as studying cells that are stimulated with LPS. It is also acknowledged that for these experiments only three samples per treatment were studied. However, the principal component analysis and statistical treatment of the data give confidence that the work was adequately powered.

The experiments generated data that was clearly different to other studies which have shown that the expression of eNOS and ET-1 are influenced by high magnesium concentrations. While this may be considered a limitation of the study, the concentrations used in the present studied (up to 5 mM) were chosen to be within reasonable physiological limits.

4.9 Conclusion

This chapter took a two-pronged approach to examining the impact of varying magnesium concentration on mRNA and protein expression in HUVECs. This involved following a candidate gene approach focused on specific endothelial cell functions, and a whole genome-wide approach using a microarray. The candidate gene approach demonstrated that low magnesium had the expected effect of increasing the mRNA and protein expression of TRPM-7. This indicates that the HUVECs model under study is robust. In contrast, none of the magnesium concentrations had a significant effect on the expression of eNOS and ET-1, which although in contrast to other studies, suggests that any cardiovascular effects of magnesium may be mediated by other mechanisms. The microarray analysis demonstrated the effects of different magnesium concentrations on global gene expression in HUVECs. Magnesium significantly regulated 12.3% of the HUVECs genome, and involved many functional groups. Most notable among the affected groups were cellular process, cell proliferation, cell killing, ligand -regulated transcription factor activity, translation -regulator activity, antioxidant activity and enzyme -regulator activity. The results also illustrated that magnesium treatments can affect the chemokine and cytokine signalling pathway. High-magnesium treatments significantly decreased the expression of IL8, IL5, IL33, IL6R, IL1RL1, IL31RA, GCP-2, GROα and GROβ. However, the low-magnesium treatments significantly down-regulated expression of IL4, CCL4,

CXCR4 and CCRL2. Low and high Mg largely had very distinct effects which included impacts on cytokine signalling, cell proliferation and other cellular processes, they also had some effects in common including cytoskeletal modelling, anion transport and protein binding. The data has raised some interesting further questions, including whether magnesium effects on inflammatory processes may provide a basis for the association between magnesium and cardiovascular function.

5

The Effect of Magnesium on the Inflammatory Response in HUVECs

5 The Effect of Magnesium on the Inflammatory Response in HUVECs

5.1 Introduction

Magnesium status may impact on cardiovascular health through a number of mechanisms (De Baaij et al., 2015). Previous work suggests that low extracellular magnesium concentrations have a negative impact on endothelial cell proliferation, increase monocyte adhesion, inhibit cell migration and markedly alter endothelial cell gene expression. Low magnesium levels contribute to inflammation. These effects may be due to the activation of many types of cytokines, which induce an overexpression of the inflammatory phenotype in endothelial cells (Maier, 2012).

Inflammation plays an essential role in atherosclerosis, ischemic injury and thrombosis (Zhang et al., 2010). In atherosclerosis, sclerotic plaques develop in the walls of the arteries in response to the dysfunction of endothelial cells, which upregulate cytokine and adhesion molecules that recruit T-cells and drive monocytes and macrophages to the subendothelial space (Maier, 2003). This results in activation of the proliferation and migration of smooth muscle cells, triggering platelet aggregation and increasing LDL oxidation (Maier et al., 2004b). Activated NF-κB plays a key role in the development of inflammation because when NF-κB transfers to the nucleus and binds to promoter regions, it initiates the transcription of many inflammatory mediators (Sun and Andersson, 2002). In response to inflammation, NF-κB enhances the transcription of ICAMs and VCAMs, and inflammatory cytokines aid in the transmigration of leukocytes from the blood vessel lumen through the barrier of the endothelial cells and into the subendothelial space (Peters et al., 2003).

Magnesium deficiency is proposed to mediate the expression of inflammatory cytokines by affecting the activity of NF-κB through TLR-2 and TLR-4 (Sugimoto et al., 2012). In contrast, magnesium has anti-inflammatory and antioxidant effects, and it has a defensive

role in triggering the body's immune cells (Liu et al., 2013). Moreover, magnesium enhances endothelial function and inhibits atherosclerosis (Ferrè et al., 2010).

5.2 Toll-like Receptor 4 Signalling Pathway

The activation of the cell surface receptor TLR-4 signalling pathway plays a critical role in inflammation as it leads to upregulation of inflammatory cytokines (Tapping et al., 2000) and cell adhesion molecules, in response to LPS (Sawa et al., 2008). This pathway is shown in Figure 5.1. As seen, the TLR-4 signalling pathway induces the NF-κB pathway. Beginning with TLR-4, activation by LPS, signals progress via the myeloid differentiation protein 88 (MyD88). This then stimulates IL-1 receptor-associated kinase-4 (IRAK-4), resulting in activation of TNF receptor-associated factor 6 (TRAF6), which leads to the activation of I kappa Kinase (Ikk). The activation of Ikk drives upregulation of NF-κB and expression of a variety of inflammatory cytokines and cell adhesion molecules (Olivieri et al., 2013).

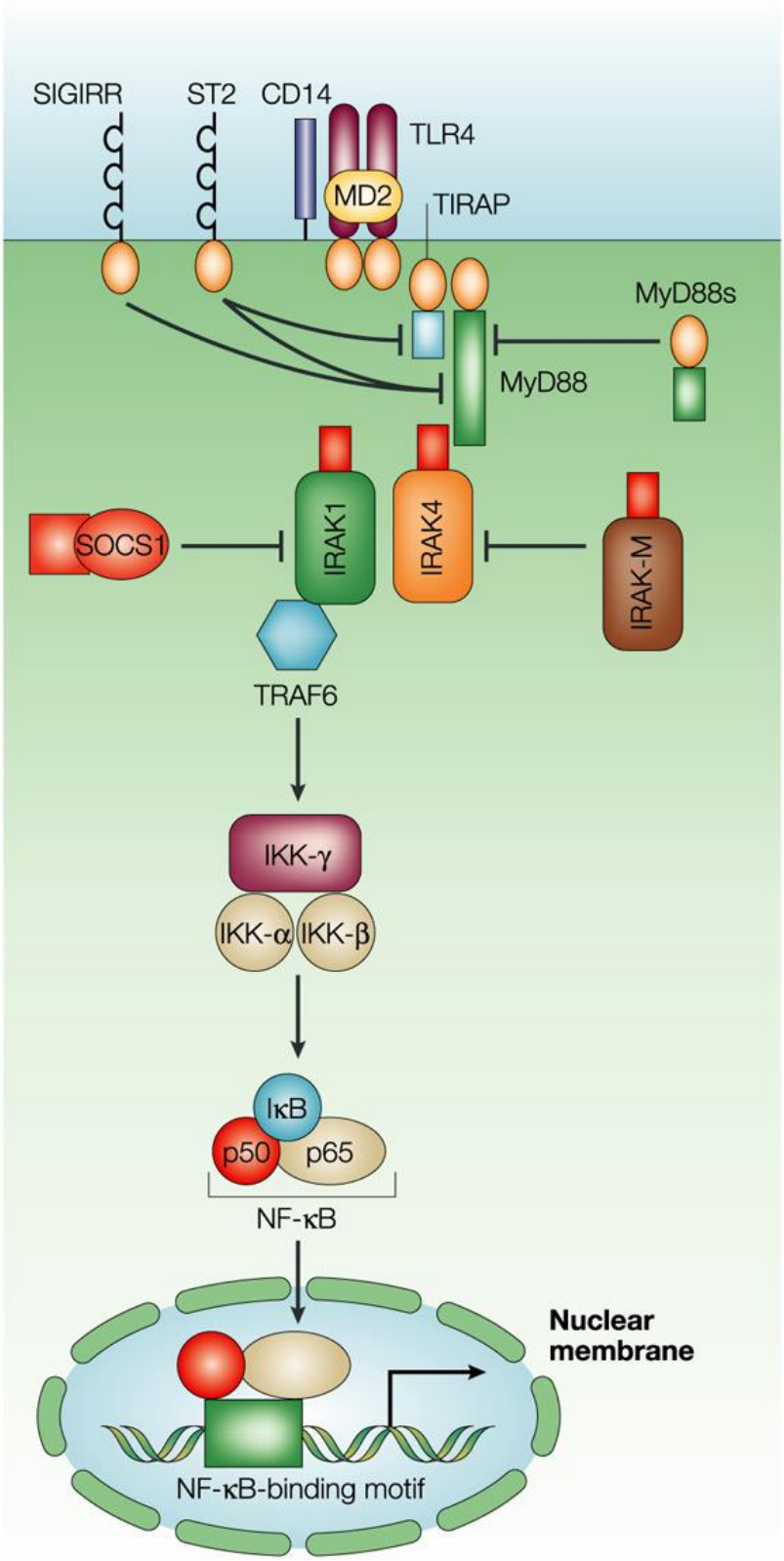


Figure 5.1 Toll-like receptor 4 signalling for the inflammation response. The red blocks indicates the phosphorylation (Akira and Takeda, 2004).

5.3 Hypothesis

In this study, it was hypothesized that magnesium regulates the expression of adhesion molecules and cytokines via the NF-κB pathway on HUVEC cells. In addition, this pathway may activate the TLR-4 signalling pathway thus increasing the expression of adhesion molecules and inflammatory cytokines in response to LPS stimulation.

5.4 Aim

Data presented in previous chapters have shown that magnesium concentrations can modify gene expression in numerous pathways, including those related to inflammatory responses. To explore the mechanism by which inflammatory responses to LPS are dependent upon magnesium, this study aimed to assess the effect of different concentrations on the expression of components of the NF-κB signalling pathway. The resulting impact on the expression of adhesion molecules and essential cytokines, in endothelial cells under basal conditions and in response to an inflammatory challenge, was also investigated. Finally, the role of magnesium in regulating the response to LPS binding to TLR-2 and TLR-4 receptor was investigated.

5.5 Objectives

- To investigate the effect of different concentrations of magnesium on expression of genes participating in the NF-κB pathway.
- To determine the effect of different concentrations of magnesium on the expression of intercellular adhesion molecules.
- To examine the effect of different concentrations of magnesium on the expression of cytokines.
- To investigate the contribution of TLR-2 and TLR-4 to effects of magnesium on LPS signalling in HUVECs.

5.6 Material and Methods

A full description of the techniques used in these experiments is presented in the Materials and Methods section (Chapter 2). A brief overview is provided in this chapter, as follows.

5.6.1 Cell Culture

HUVECs were cultured in 6-well plates (for adhesion assay, a 24-well plate was used) in EGM-2 with 2% FBS. The cells were then incubated at 37°C (5% CO_2). At 80% confluence, the HUVECs were cultured in magnesium-free human endothelial medium (Invitrogen, USA) supplemented with $MgSO_4$ concentrations of 0.1 mM and 5 mM, and then the samples were compared to the physiological circulating concentration of $MgSO_4$ (1 mM) (see section 2.1.9). After 3 days, the cells were treated with LPS (0.5 µg/ml) for 4 hours, and the response compared to untreated cells (see section 2.1.10). Data in this chapter was obtained from 8 separate experiments as shown in Table 5.1.

Table 5.1 Experiments reported in this chapter

Experiment	Outcome measures
Experiment 1	NF-κB, adhesion molecule, cytokines gene expression
Experiment 2	NF-κB pathway expression
Experiment 3	Adhesion assay
Experiment 4	ICAM western blot analysis
Experiment 5	VCAM ELISA
Experiment 6	Cytokine protein expression
Experiment 7	TLR2-4 blocking assay gene expression
Experiment 8	TLR2-4 blocking assay protein expression (IL-8/ VCAM ELISA)

5.6.2 Measurement of mRNA by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. Single-stranded cDNA was synthesised using a ReverseAid RT Kit (Thermo). qRT-PCR was performed using a Roche LightCycler 480, with a cycling state of 95°C for 5 minutes, then 45 cycles for 10 seconds at 95°C, followed by 30 seconds at 60°C, using a SYBR Green Master Mix. The values were normalised to the cyclophilin housekeeping gene (see section 2.3).

5.6.3 Western Blot Analysis

The cells were lysed in 120 µl RIPA buffer with protease inhibitor cocktails. The samples were denatured at 90°C for 5 minutes. Then, 30 µg of protein was loaded into each well in 4-10% mini-protean TGX precast polyacrylamide gradient gel. Next, the samples were transferred to a nitrocellulose membrane, and then the membrane was blocked. The proteins were detected by using a primary antibody anti-ICAM (Abcam), followed by a secondary antibody to detect the primary antibody. The membranes were then impregnated with enhanced chemiluminescence (ECL) detection (Prime Western Blotting Detection Reagent Kit, GE Healthcare) and visualised by X-ray film exposure (see section 2.5).

5.6.4 Human Cytokine Array

The protein concentration of several cytokines in lysed cells was measured using a Human Cytokine Antibody Array Membrane Kit (Abcam), according to the manufacturer's instructions. The spot signal densities were analysed using Quantity One Analysis Software (Bio-Rad) (see section 2.6).

5.6.5 NF-κB Pathway Array

The concentration of a number of proteins of NF-κB pathway activity in lysed cells was determined using a Human NFκB Pathway Array Kit (R&D Systems, Ltd.), according to the manufacturer's instructions. The spot signal densities were analysed using Quantity One Analysis Software (Bio-Rad) (see section 2.7).

5.6.6 TLR-2 and TLR-4 Blocking Assay

TLR-2 and TLR-4 blocking antibodies (10 μg/mL) (InvivoGen, USA) were added for 30 minutes before stimulating the cells with 0.5 μg/ml LPS for 4 hours. The culture media was collected for ELISA, and the cells were collected for RNA extraction (see section 2.8).

5.6.7 ELISA

After incubating the HUVEC cells with LPS for 4 hours, the culture media were collected to measure IL-8 and VCAM using a Human CXCL8/IL-8 Quantikine Immunoassay Kit and a Human VCAM-1 DuoSet Kit (R&D Systems, Ltd.) (see section 2.9).

5.6.8 Cell Adhesion Assay

To assess monocyte adhesion, human monocytic THP-1 cells were labelled with 5 μM of calcein for 30 minutes. The calcein-labelled cells were co-cultured with the HUVECs, and then incubated for 60 minutes. Relative fluorescence was used as an index of adhesion (see section 2.10).

5.7 Results

5.7.1 Effect of Different Concentrations of Magnesium and LPS on the Expression of the Genes and Proteins Participating in the NF-κB Pathway

5.7.1.1 NF-κB Pathway Gene Expression

To determine the effect of magnesium on the genes involved in the NF-κB pathway, the HUVEC cells were cultured as described in 5.6.1 (experiment 1). LPS stimulation of TLR-2 and TLR-4 activates the NF-κB pathway (Hippenstiel et al., 2000), and downstream signalling is induced by exposure to LPS (Sugimoto et al., 2012). To investigate any effect of magnesium, the mRNA expression of TLR-2, TLR-4, IRAK, TRAF6, IkB, NF-κB and P65 were analysed by qRT-PCR.

The results confirmed that HUVECs expressed TLR-2 and TLR-4. As shown in Figure 5.2 B, there was significant effect of magnesium concentration and LPS on TLR4 mRNA expression (P =0.001 for magnesium concentration × LPS interaction), and a low concentration of magnesium markedly increased the TLR-4 mRNA relative to the physiological concentration of magnesium 1 mM, in the presence of LPS. Magnesium concentration did not significantly alter the TLR-2 mRNA expression (Figure 5.2A). In the absence of LPS magnesium concentration had no effect on expression of TLR-2 or TLR-4.

The data showed that, in high and low magnesium concentrations, the IRAK-1 mRNA and TRAF-6 mRNA was not significantly LPS x Mg interaction. Moreover, LPS stimulation did not significantly alter the IRAK-1 mRNA in the HUVECs cells (Figure 5.2 C, D).

As shown in Figure 5.3B, a significant increase in NF-κB2 mRNA was observed in the magnesium-deficient cells that were stimulated with LPS (P=0.032, Dunnett test), Moreover, a marked decreased in NF-κB2 mRNA in the magnesium-treated stimulated HUVECs was observed (P=0.048, Dunnett test) relative to the 1 mM physiological concentration (Figure 5.3 B). Analysis, showed that the magnesium treatment had no effect on P65 and IKB mRNA concentration (Figure 5.3A & C).

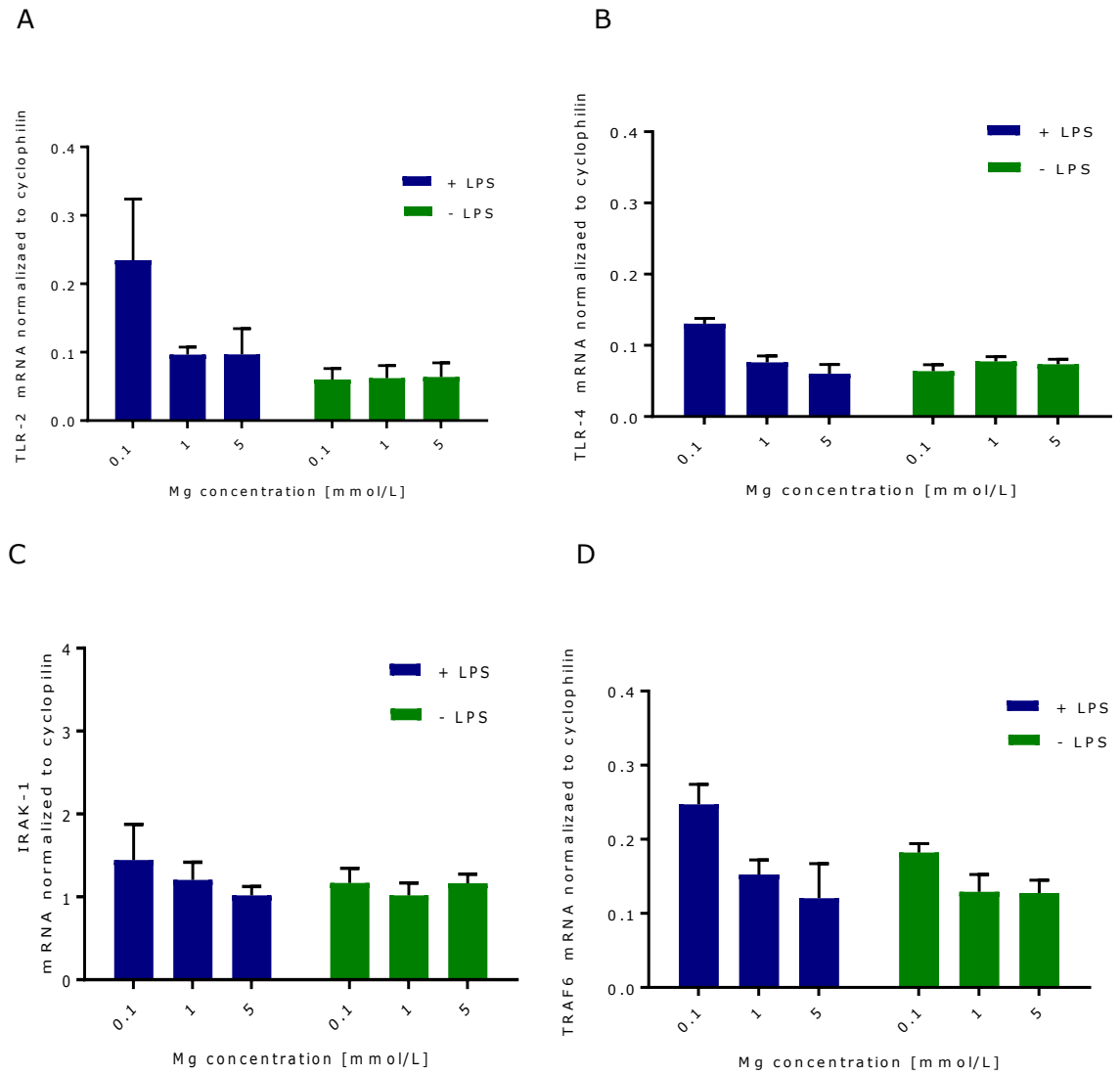


Figure 5.2 Effect of different concentrations of magnesium on NF-κB pathway gene expression. Confluent HUVECs after 3 days of culture with $MgSO_4$ (0.1 mM, 1 mM, 5 mM) stimulated with or without LPS (0.5 μg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data is shown as Mean±SEM for n=4. A: Expression of TLR-2 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*=0.2). There was a significant effect of LPS (*P*= 0.039), no significant effect of Mg (*P*= 0.2). B: Expression of TLR-4 mRNA. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (*P*=0.001). C: Expression of IRAK1 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*=0.65). There was no significant effect of LPS (*P*= 0.59) or Mg (*P*= 0.6).D: Expression of TRAF6 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*=0.42). There was a significant effect of LPS (*P*= 0.012), no significant effect of Mg (*P*= 0.23).

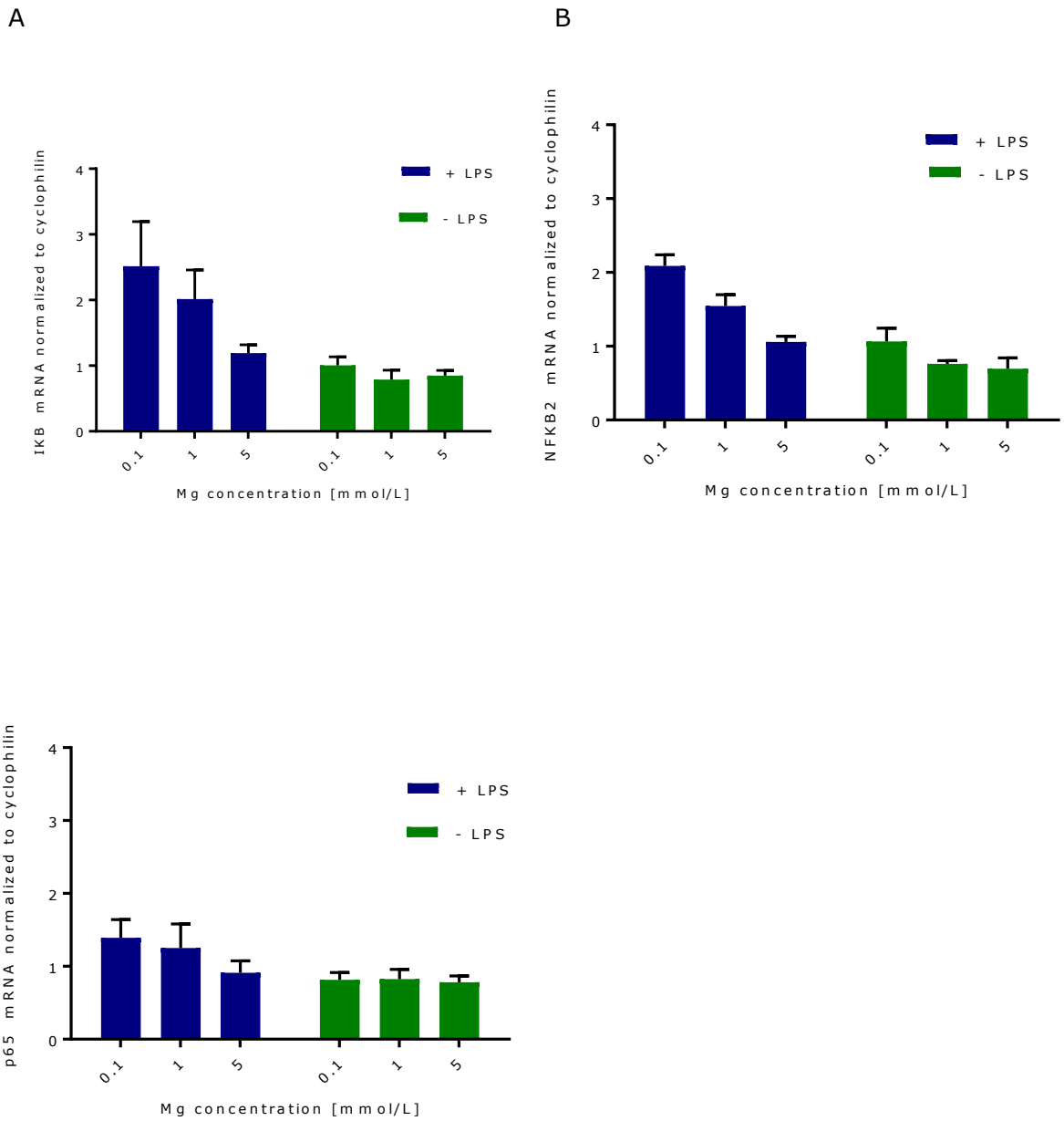


Figure 5.3 Effect of different concentrations of magnesium on NF-κB pathway gene expression. Confluent HUVECs after 3 days of culture with $MgSO_4$ (0.1 mM, 1 mM, 5 mM) stimulated with or without LPS (0.5 μg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data is shown as Mean± SEM for n=4. A: Expression of IKB mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (P=0.2). There was a significant effect of LPS (P= 0.002), no significant effect of Mg (P= 0.15). B: Expression of NF-KB 2 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (P=0.07). There was a significant effect of magnesium concentration (P<0.001) and LPS (P< 0.001). C: Expression of P65 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (P=0.57). There was a significant effect of LPS (P= 0.039), no significant effect of Mg (P= 0.47).

5.7.1.2 NF-κB Pathway Protein Concentration

In an attempt to gain more detailed understanding of the NF-κB pathway's response to magnesium, HUVEC cells were cultured in different magnesium concentration as described in section 5.6.1 and the cells were then treated with LPS (0.5 µg/ml) for 4 hours (experiment 2). Protein extraction was performed and concentration of proteins in the pathway was assessed using the NF-κB array kit. The array considered expression of 41 proteins, but for this analysis we focused on the 13 proteins involved in inflammatory processes TLR-2, MYD88, TRAF3 , IRAK1, Ikkα, Ikkβ, Ikkγ, IkBα, IkBε, NF-κB 1, NF-κB 2, RelA and c-Rel as shown in Figure 5.4 .

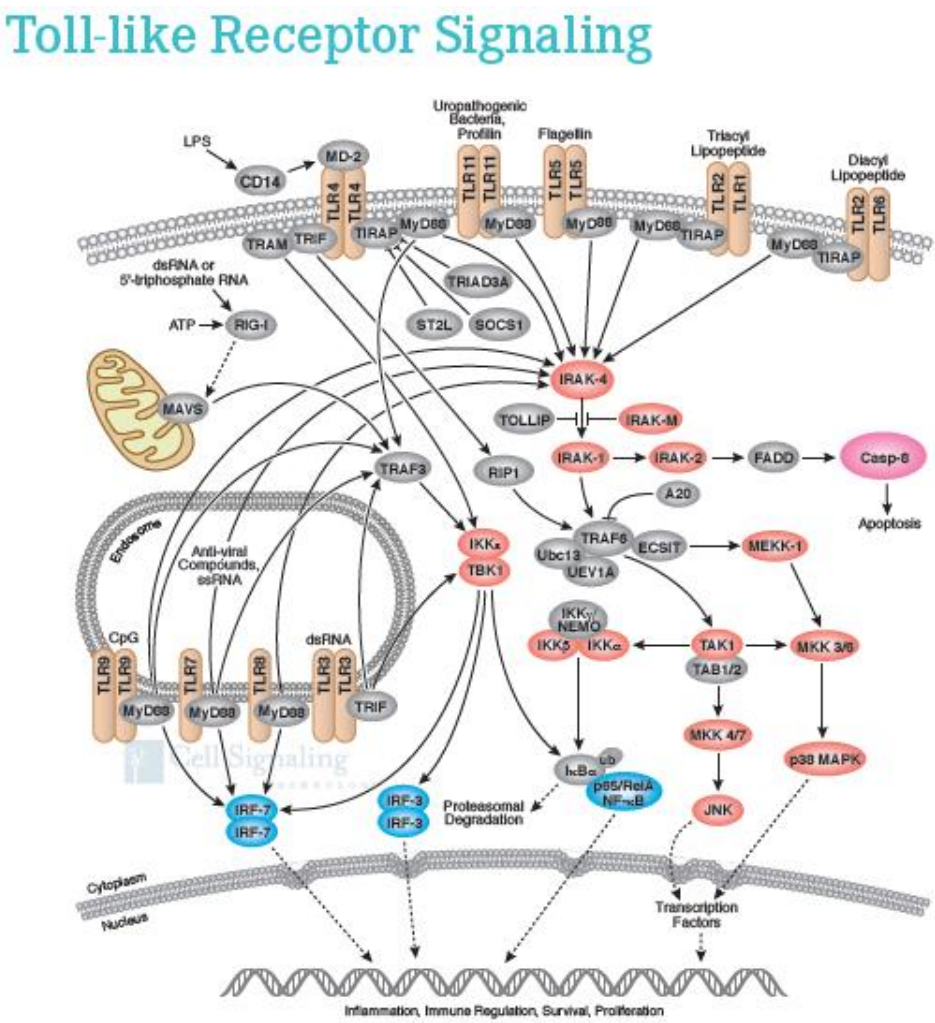


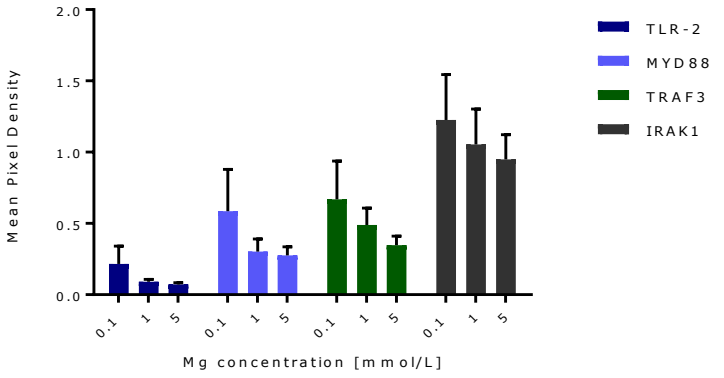
Figure 5.4 Toll-like receptor signalling for NF-κB activation. (https://www.cellsignal.com/common/content/content.jsp?id=pathwaystlr&pathway=Toll-Like ReceptorSignaling)

Under LPS stimulation, there were no significant effects of either low or high magnesium upon concentration of TLR2 (*P*= 0.36), MyD88 (*P*= 0.43), TRAF3 (*P*= 0.45) and IRAK1 (*P*= 0.74) proteins. There were, however, some non-significant trends with a general pattern for magnesium deficient cells to express the proteins at a higher level than high-magnesium (Figure 5.5 A).

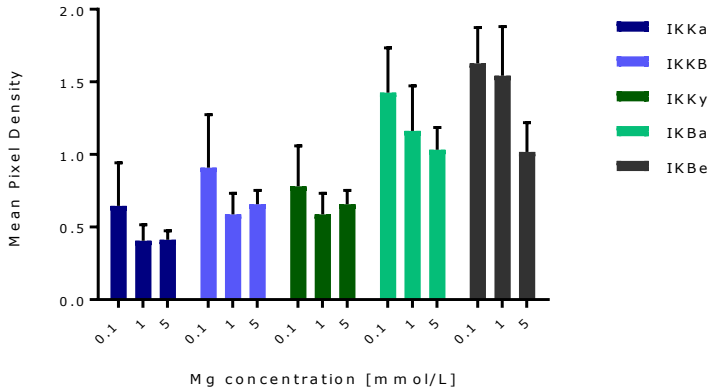
We then determined how Mg affected the sequential recruitment of proteins in the signalling pathway. The result clarified that low magnesium did not affect signalling via the MyD88-independent pathway Ikkα (*P*= 0.59), Ikkβ (*P*= 0.6), Ikkγ (*P*= 0.76), IkBα (*P*= 0.58) and IkBε (*P*= 0.27) (Figure 5.5B).

These findings indicate that Mg insufficiency did not significantly change the NF-κB2 (*P*= 0.42) and P65 (*P*= 0.49) protein levels following LPS treatment, in comparison to the control. Further, a high concentration of magnesium did not affect the expression of these proteins. The protein concentration pattern was similar to the mRNA expression pattern. However, no modulation of the concentration of NF-κB1 (*P*= 0.61) and c-Rel (*P*= 0.58) proteins was observed (Figure 5.5C).

A



B



C

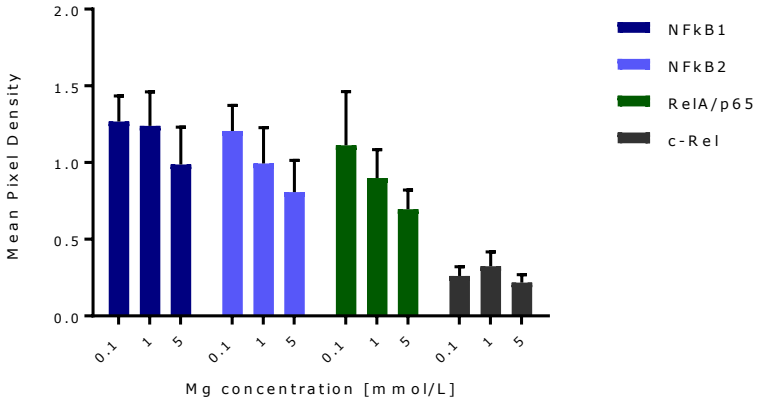


Figure 5.5 Effect of different concentrations of magnesium on NF-κB pathway protein concentration. Confluent HUVECs after 3 days of culture with $MgSO_4$ (0.1 mM, 1 mM, 5 mM) stimulated with LPS (0.5 μg/ml) for 4 hours. Protein extracts were assayed using an NF-κB Pathway Kit. Data is shown as Mean± SEM for n=4. A: One way ANOVA showed no significant effect of magnesium concentration on TLR-2, MYD88, TRAF3 and IRAK1 protein concentration. B: One way ANOVA showed no significant effect of magnesium concentration on Ikkα, Ikkβ, Ikkγ, IkBα and IkBε protein concentration. C: One way ANOVA showed no significant effect of magnesium concentration on NF-κB 1, NF-κB 2, RelA and c-Rel protein concentration.

5.7.2 Effect of Different Concentrations of Magnesium and LPS on the Expression of Intercellular Adhesion Molecules

5.7.2.1 Adhesion Assay

To evaluate the impact of magnesium on the binding of THP-1 cells to HUVECs, these cells were cultured for 72 hours in medium containing 0.1 mM, 1 mM and 5 mM $MgSO_4$. The cells were then exposed, or not, to 0.5 μg/ml LPS, and the resulting interaction was evaluated via binding of fluorescence labelled THP-1 cells (experiment 3).

The current study found that there was a significant interaction between the effects of magnesium concentration and presence of LPS on the adhesion of THP-1 cells (P= 0.044). Generally this interaction was being clearly in high magnesium treatment cells. In the present and absence of LPS, the adhesion of THP-1 cells was significantly higher when HUVECs were cultured in the low magnesium concentration of 0.1 mM compared to the control group 1 mM. In contrast, treating HUVECs with 5 mM magnesium significantly inhibited the adhesion of THP-1 cells to HUVECs compared to the 1 mM dose (Figure 5.6). The increase in THP-1 binding to HUVECs occurred when the NF-κB was activated by way of the low concentration (0.1 mM) of magnesium. In contrast, the decrease in THP-1 binding to HUVECs occurred when the NF-κB was inhibited by way of the high concentration (5 mM) of magnesium.

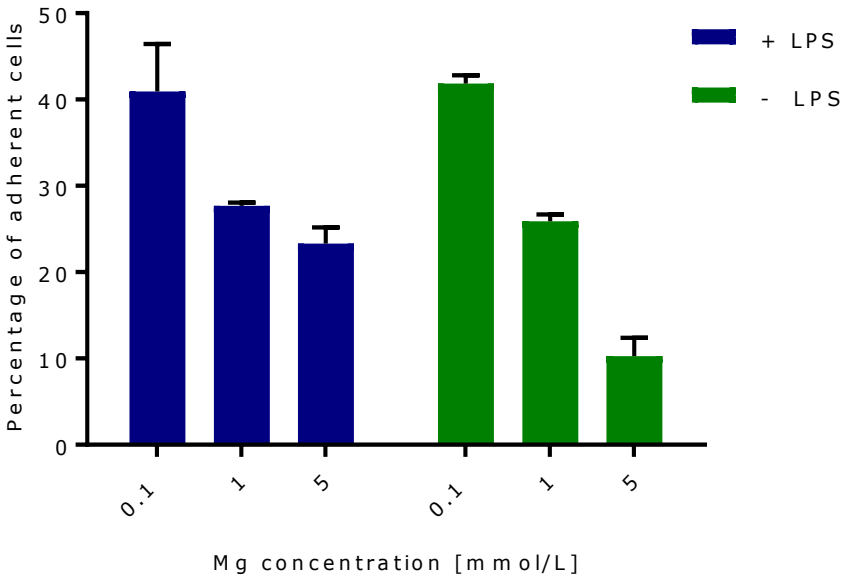


Figure 5.6 THP-1 adherence to HUVECs. Confluent HUVECs after 3 days in a culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) stimulated, with or without LPS (0.5 μg/ml) for 4 hours. Fluorescence labelled THP-1 were added to the cultured HUVECs for one hour. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P = 0.044).

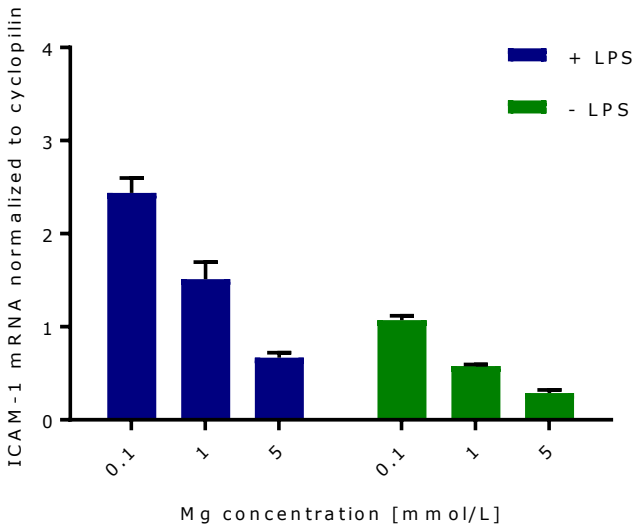
5.7.2.2 Adhesion Molecule mRNA Expression

To assess the effect of low magnesium on adhesion molecule mRNA expression in HUVECs (experiment 1), cells were cultured in different magnesium concentration as described in 5.6.1. The result showed a significant interaction of magnesium concentration and present of LPS on ICAM-1 mRNA and VCAM-1 mRNA (P=0.001, P<0.001 respectively).

Following the treatment of cells with LPS to induce an inflammatory response, both ICAM-1 and VCAM-1 mRNA was significantly increased in the presence of low magnesium, compared to the physiological concentration 1 mM. In contrast, the higher magnesium concentration significantly reduced the mRNA for both ICAM-1 and VCAM-1, relative to 1 mM controls (Figure 5.7 A & B). Low magnesium (0.1 mM) upregulated ICAM-1, and 5 mM magnesium significantly decreased its expression in unstimulated HUVECs (Figure 5.7 A). This study found no difference in VCAM-1 expression in unstimulated cells (Figure 5.7 B) when comparing low or high magnesium with the physiological concentration.

Based on these findings, it is clear that magnesium insufficiency induces the expression of cell surface adhesion molecules and that magnesium supplementation inhibits their expression. This is consistent with the hypothesis that magnesium insufficiency is associated with an exaggerated inflammatory response.

A



B

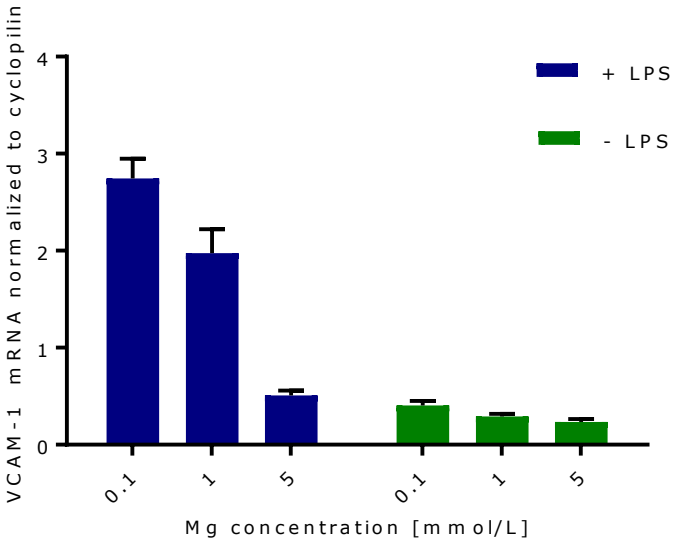


Figure 5.7 Effect of different concentrations of magnesium on adhesion molecule gene expression. Confluent HUVECs after 3 days in a culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) stimulated, with or without LPS (0.5 μg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean±SEM for n=4. A: Expression of ICAM-1 mRNA. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P = 0.001). B: Expression of VCAM-1 mRNA. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P< 0.001).

5.7.2.3 Adhesion Molecule Protein Concentrations

The effect of magnesium concentration on ICAM-1 protein was analysed by Western blotting (experiment 4), using alpha tubulin as an internal control. A clear single band was visible at ~57 KDa. Band intensity was quantified by densitometry analysis software and expressed relative to alpha tubulin. The Western blot analysis showed a significant increase in ICAM-1 protein (P=0.048) in response to low magnesium concentrations Compared the control group 1 mM (Figure 5.8 B), but no impact of high magnesium concentration. Protein expression of VCAM-1 in the media was assessed using an ELISA (experiment 5). The results showed a significant effect of magnesium concentration (P= 0.022) and presence of LPS (P = 0.024; Figure 5.9) on VCAM-1 protein concentration. VCAM-1 expression was significantly increased in magnesium deficient cells and this was enhanced by LPS stimulation (P=0.022, Dunnett test).

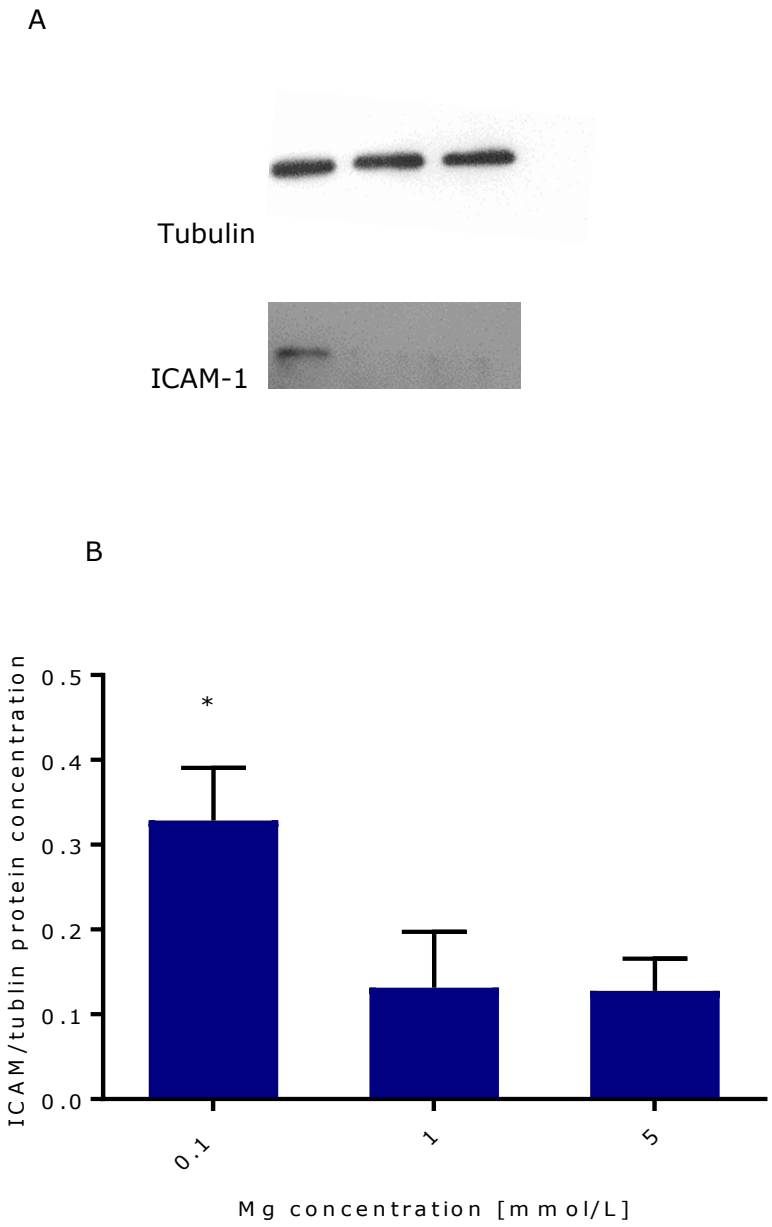


Figure 5.8 Western blot analysis of the effect of $MgSO_4$ concentration on ICAM protein levels in cultured HUVECs. The lyses cells were prepared for western blot using anti-ICAM-1 antibodies, as indicated in the Materials and Methods section of this work. The same blot was reported with anti-tubulin antibodies. A: Representative bands for ICAM-1 and tubulin. B: One way ANOVA showed significant effect of magnesium concentration (P=0.038), with a marked increase in ICAM-1 protein concentration in low magnesium concentration (P=0.048, Dunnett t). Data are shown as mean±SEM for n=3. * indicates P<0.05, compared to 1mM $MgSO_4$ in the same treatment (Dunnett t).

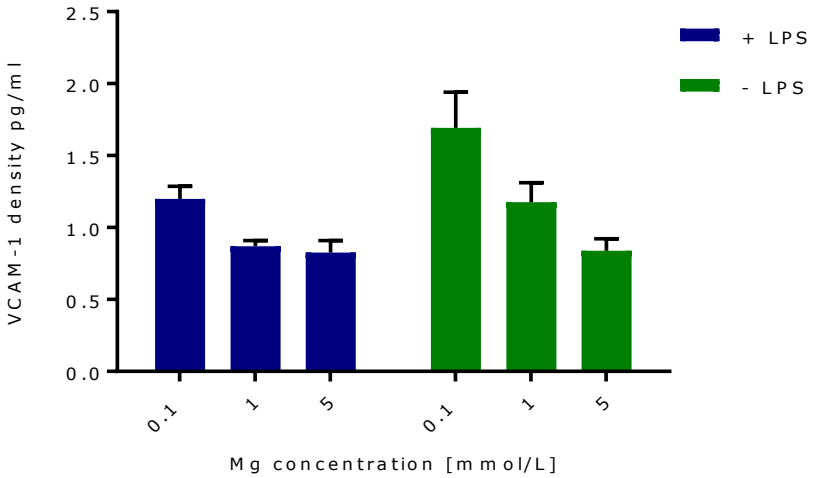


Figure 5.9 Effect of different concentrations of magnesium on VCAM-1 protein concentration. Confluent HUVECs after 3 days of culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) stimulated, with LPS (0.5 μg/ml) for 4 hours. Culture media were collected and measured for VCAM-1 production by ELISA. Data are shown as mean±SEM for n=4. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*= 0.2). There was a significant effect of magnesium concentration (*P*= 0.001), and presence of LPS (*P* = 0.021).

5.7.3 Effect of Different Concentrations of Magnesium and LPS on the Expression of Cytokines

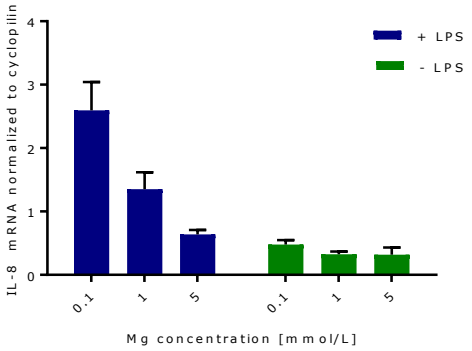
5.7.3.1 Cytokine mRNA Expression

The possibility that the gene expression of cytokines was modulated by different extracellular concentrations of magnesium was next investigated in cultured HUVECs (experiment 1).

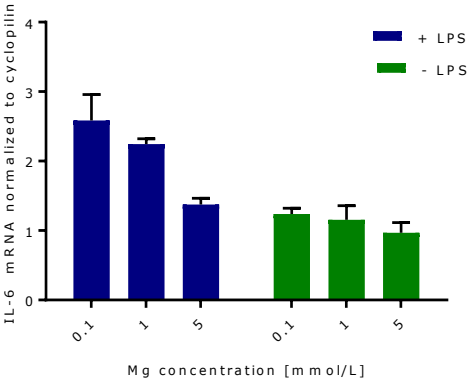
The results showed that magnesium had a significant effect on IL-8 mRNA and MCP-1 mRNA in the presence of LPS (P=0.004, P<0.001 respectively for magnesium concentration × presence of LPS interactions). Furthermore, cultured HUVECs stimulated with LPS in a 0.1 mM magnesium concentration had significantly greater mRNA concentration for IL-8 and MCP-1 relative to cells cultured in 1 mM magnesium concentration. In contrast, a marked decline in IL-8 and MCP-1 mRNA was seen at the highest magnesium concentration (5 mM) with LPS treatment compared to 1 mM magnesium concentrations (Figure 5.10 A &C).

Figure 5.10 B shows that magnesium and the presence of LPS had a significant effect on IL-6 mRNA (P=0.002, P<0.001 respectively). Interestingly, a marked decline in IL-6 mRNA (P=0.043, Dunnett test) was seen at the highest magnesium concentration 5 mM with LPS treatment compared to 1 mM magnesium concentration. On the other hand, no significant change in the expression of IL-1B and TNF-B was found between the magnesium treatment groups nor the LPS-treated group (Figure 5.11 A & B).

A



B



C

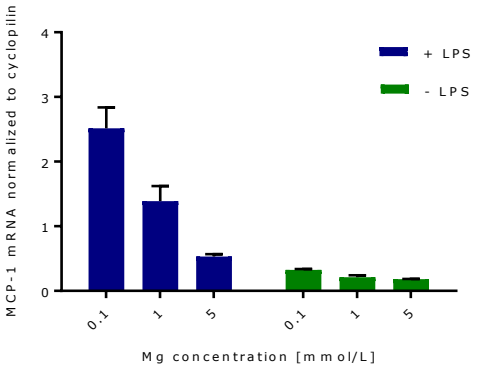
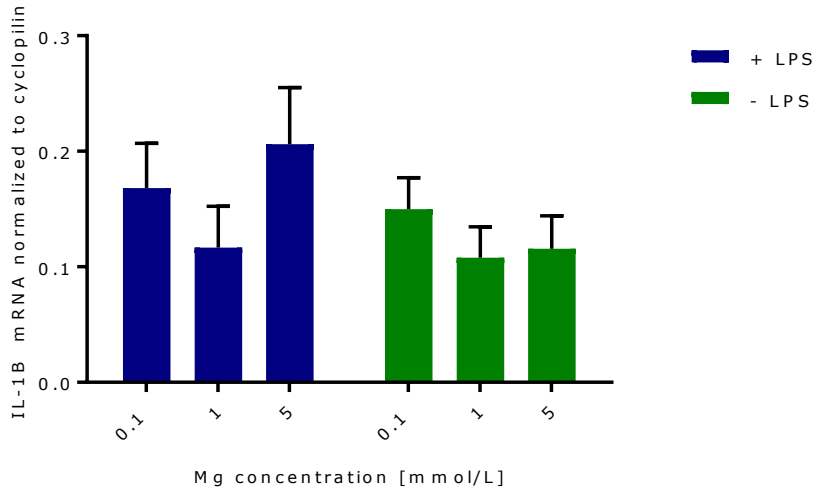


Figure 5.10 Effect of different concentrations of magnesium on cytokine gene expression. Confluent HUVECs after three days in a culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) stimulated with or without LPS (0.5 µg/ml) for four hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean ± SEM for n=4. A: Expression of IL-8 mRNA. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P= 0.004). B: Expression of IL-6 mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (P= 0.053). There was a significant effect of magnesium concentration (P= 0.002), and presence of LPS (P< 0.001). C: Expression of MCP-1 mRNA. Two-way ANOVA showed significant interaction of magnesium concentration × presence of LPS (P< 0.001).

A



B

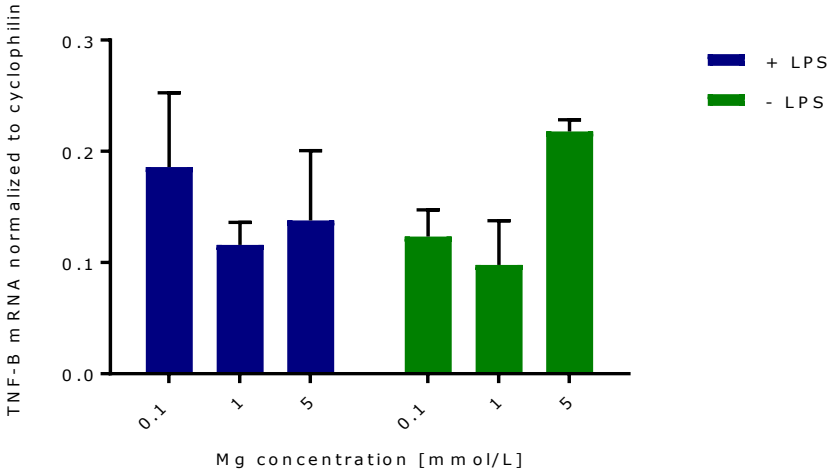


Figure 5.11 Effect of different concentrations of magnesium on cytokine gene expression. Confluent HUVECs after three days in a culture with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) stimulated with or without LPS (0.5 µg/ml) for four hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean ±SEM for n=4. A: Expression of IL-1B mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*= 0.46). There was no significant effect of magnesium concentration or presence (*P*=0.31) of LPS (*P*=0.19). B: Expression of TNF-B mRNA. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*=0.87). There was no significant effect of magnesium concentration (*P*= 0.66) or presence of LPS (*P*= 0.94).

5.7.3.2 Cytokine Protein Concentrations

Given the results that clearly showed that low magnesium induces the activation of the expression of NF-κB, which causes increased expression of many inflammation genes that drive atherosclerosis and impact upon gene expression of inflammatory cytokines, additional evidence was sought to establish that different concentrations of magnesium can modulate the protein concentration of inflammatory cytokines. HUVECs were grown as described in 5.6.1. Protein was then extracted from cells (experiment 6) and cytokine proteins were measured using the human cytokine antibody array membrane kit. The array was designed to detect 42 human cytokine proteins; for this study we concentrated on the 18 cytokine proteins expressed in HUVECs.

The results from the cytokine array membrane confirmed that magnesium insufficiency notably enhanced the expression of many cytokines from HUVECs, such as IL-2 (P=0.029), IL-3 (P=0.023), IL-8 (P=0.038), MCP-1 (P=0.029), GRO (P=0.003) and GROα (P=0.044) compared to the 1 mM magnesium concentration (Figure 5.12 A,B & Figure 5.13). Conversely, the results showed that the high magnesium concentration (5 mM) reduced the protein concentration of IL-2 (P=0.05) and IL-6 (P=0.01) (Figure 5.12 A).

The results shown in Figure 5.12 and 5.13 indicate that magnesium did not affect IL-1 and TNF protein which was consistent with the mRNA data. Additionally, no effect of magnesium was observed on the cellular concentrations of IL4, IL5, IL7, IL10, IL12, IL-13 and IL15.

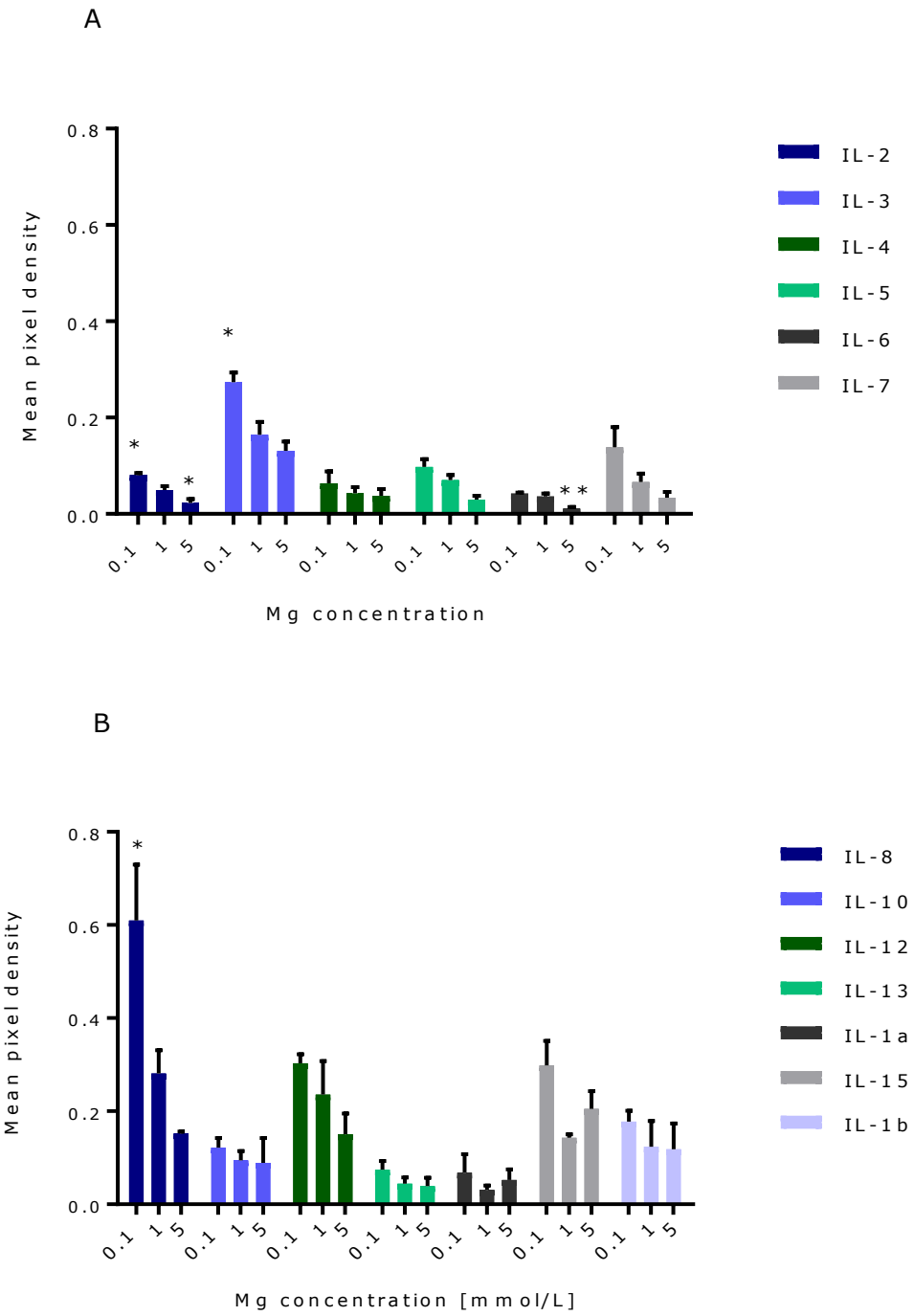


Figure 5.12 Effect of different concentrations of magnesium on cytokine array protein concentration. Confluent HUVECs after 3days of culture with $MgSO_4$ (0.1, 1, 5 mM) stimulated with LPS (0.5µg/ml) for 4h. Protein extracts were assayed by the human cytokine antibody array membrane kit. Data are shown as mean ± SEM for n=3. * indicates $p<0.05$, ** indicates $p<0.01$ compared to 1mM $MgSO_4$ in same treatment (Dunnett t). A: Protein concentration of IL-2, IL-3, IL-4, IL-5, IL-6 and IL-7. One way ANOVA showed a significant effect of magnesium concentration on IL-2 ($P=0.003$), IL-3 ($P=0.009$) and IL-6 ($P=0.004$). B: Protein concentration of IL-8, IL-10, IL-12, IL-13, IL-1a, IL-15 and IL-1b. One way ANOVA showed a significant effect of magnesium concentration on IL-8 ($P=0.013$).

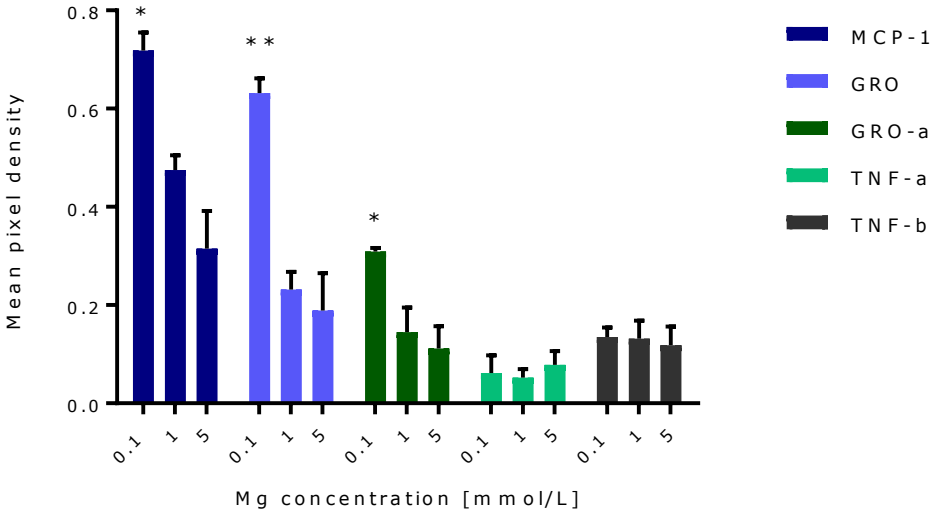


Figure 5.13 Effect of different concentrations of magnesium on cytokine array protein concentration. Confluent HUVECs after 3days of culture with $MgSO_4$ (0.1, 1, 5 mM) stimulated with LPS (0.5µg/ml) for 4h. Protein extracts were assayed by the human cytokine antibody array membrane kit. Data are shown as mean ± SEM for n=3. * indicates $p<0.05$, ** indicates $p<0.01$ compared to 1mM $MgSO_4$ in same treatment (Dunnett t). Protein concentration of MCP-1, GRO, GRO-α, TNFa and TNFb. One way ANOVA showed a significant effect of magnesium concentration on MCP-1 (P=0.004), GRO (P= 0.002) and GROα (P=0.025).

5.7.4 Contribution of TLR-2 and TLR-4 in the Lipopolysaccharide (LPS) Signalling in HUVECs

5.7.4.1 Effect of blocking TLR-2 and TLR-4 on mRNA for genes associated with inflammation

The results presented earlier in the thesis and in this chapter have suggested that varying magnesium concentrations impacts upon expression of inflammatory pathways and modifies the response to LPS, with an increased inflammatory response in magnesium deficiency. As LPS signalling is mediated by TLRs the aim of this experiment (experiment 7) is to establish whether TLR-2 or TLR-4 mediated signalling is sensitive to magnesium. HUVECs were treated with various concentrations of magnesium for 72 hours. Then, cells were pre-treated for 30 minutes in five treatment groups: anti-TLR-2; anti-TLR-4; anti-TLR-2 and anti-TLR-4 together; cells not blocked and used as a control; and cells not blocked and not stimulated. Finally, four of the five treatment groups were stimulated with LPS for 4 hours. At the end of the incubation period, the cells were collected, RNA was extracted, and qRT-PCR was performed to analyse the potential of TLR-2 and TLR-4 antibodies in blocking the inflammatory gene's expression. The rationale for this was that if TLR blockade nullified the effect of magnesium on expression of a particular gene, then that TLR must play a role in mediating the magnesium effect.

Two way ANOVA showed a significant interaction between magnesium concentration and LPS/blocker on NF-κB mRNA ($P<0.001$). Generally this interaction was explained by the effect of LPS being greater in magnesium insufficient cells than in cells with high magnesium. The results of the experiment showed that blocking LPS-stimulated HUVECs cultured in 0.1 mM $MgSO_4$, significantly decreased the NF-κB mRNA after incubation with anti-TLR-2, anti-TLR-4 and both anti-TLR-2+TLR-4 antibodies when compared to the control group at the same magnesium concentration. Moreover, the HUVECs that were cultured in 1 mM $MgSO_4$ and then blocked and LPS-stimulated also showed a marked decline in the NF-κB mRNA in the cells that were blocked with anti-TLR-2, anti-TLR-4 and both anti-TLR-2 + TLR-4 compared to 1 mM in the group not treated with blocking

antibodies. As expected, a high magnesium concentration (5 mM) markedly reduced NF-κB mRNA in LPS-stimulated HUVECs without blocking compared to the HUVECs cultured in 1 mM $MgSO_4$ of the same treatment group. In addition, NF-κB mRNA decreased considerably in the cells which were cultured in 0.1 mM $MgSO_4$ and 1 mM $MgSO_4$ that were not blocked and not stimulated compared to the cells at the same magnesium concentration in the control group (Figure 5.14 A).

ICAM-1 mRNA was also significantly affected by magnesium concentration and LPS/blocker interaction ($P<0.001$). The main driver of ICAM-1 expression was LPS, but the effects were altered by magnesium concentration. LPS had a greater impact in magnesium insufficiency. The result showed that the stimulated HUVECs that were cultured in 0.1 mM $MgSO_4$ and blocked by anti-TLR-2, anti-TLR-4 and both anti-TLR-2+TLR-4 antibodies demonstrated notably lower ICAM-1 mRNA, compared to the same magnesium concentration in the control group. Furthermore, anti- TLR-4 and anti-TLR-2 + TLR-4 antibodies significantly decreased the ICAM-1 mRNA in the LPS-stimulated HUVECs that were cultured in 1 mM $MgSO_4$ compared control cells. The blockade experiment showed that the effects of LPS in the magnesium insufficient cells were mainly via the TLR4 receptor, with a lesser role for TLR2. Another important finding was that a 5 mM $MgSO_4$ concentration markedly decreased the ICAM-1 mRNA in HUVECs after treatment with LPS. Similarly, HUVECs that were grown at a high magnesium concentration and blocked with anti-TLR-4 showed a decrease in ICAM-1 mRNA compared to cells at the same magnesium concentration in the control group. The study found that the levels of ICAM-1 mRNA notably decreased in the HUVECs that were unblocked, unstimulated, and cultured in 0.1 mM $MgSO_4$ and 1 mM $MgSO_4$ compared to the same magnesium concentration in the control group. In contrast, HUVECs that were cultured in 0.1 mM $MgSO_4$ and then stimulated significantly increased ICAM-1 mRNA compared to 1 mM in the same treatment group (Figure 5.14 B).

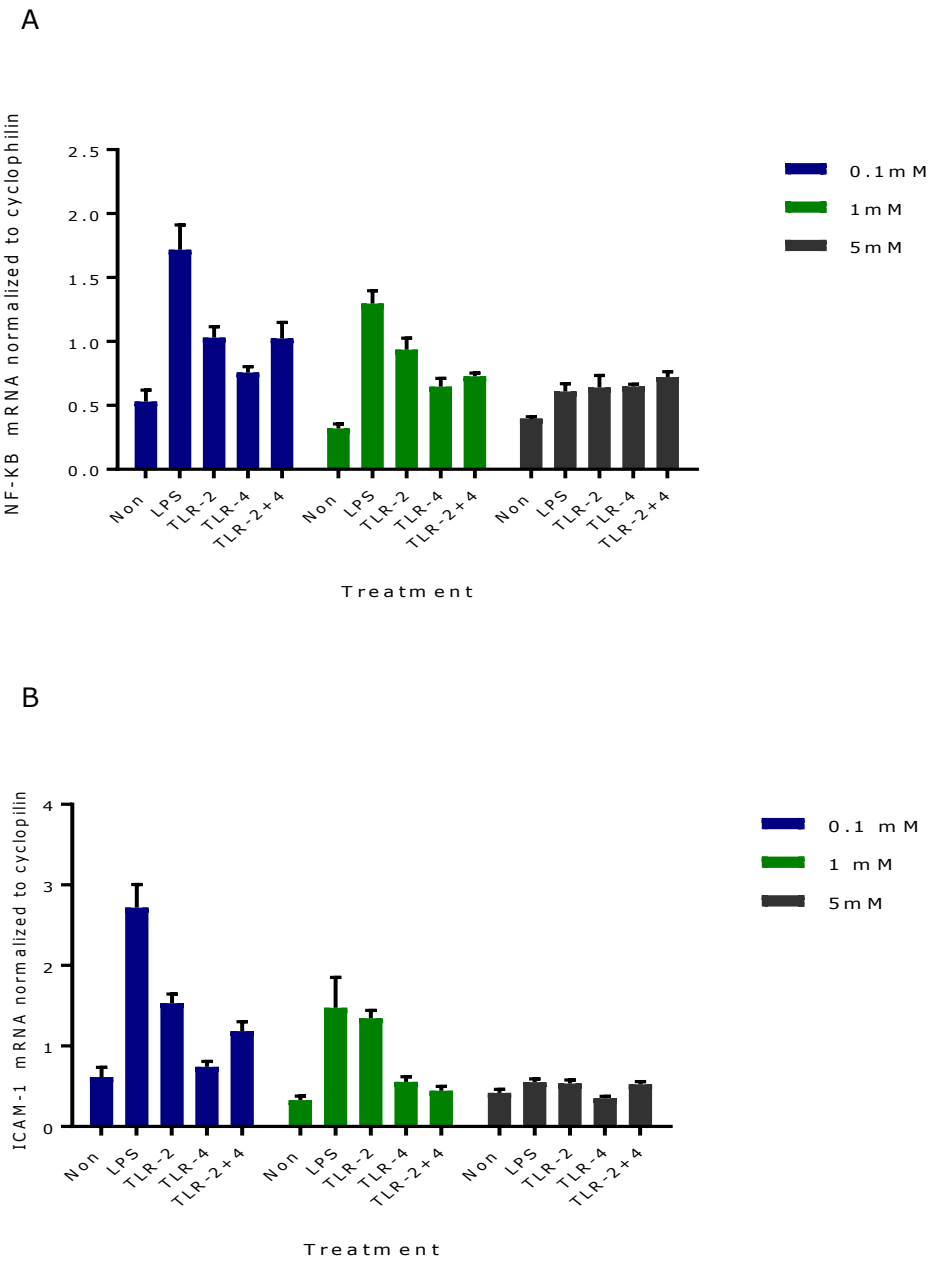


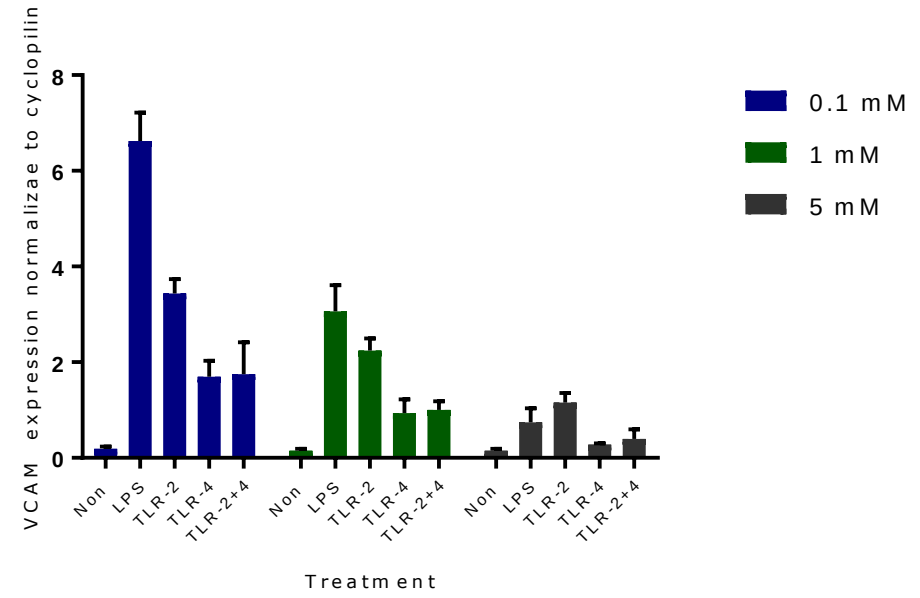
Figure 5.14 Effect of different concentrations of magnesium on HUVECs gene expression when treated or not treated with LPS and TLR antibodies. Confluent HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for three days, and then, TLR-2 and TLR-4 blocking antibodies (10µg/ mL) were added to the HUVECs for 30 minutes before being stimulated, with or without LPS (0.5 µg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean ±SEM for n=4. A: Expression of NF-κB mRNA. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker ($P< 0.001$). B: Expression of ICAM-1 mRNA. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker ($P< 0.001$).

There was a significant interaction between magnesium concentration and LPS/blocker on VCAM-1 mRNA (P<0.001). As with ICAM-1 the main effect of treatment on expression was due to LPS treatment. This was greatest in low magnesium conditions. The results indicated that blocking TLR-2 and TLR-4 receptors in the HUVECs that were treated with a low magnesium concentration followed by LPS stimulation greatly reduced VCAM-1 mRNA compared to 0.1 mM in the control group. Likewise, stimulated HUVECs cultured in the physiological concentration of magnesium 1 mM and then anti-TLR-2 and TLR-4 antibodies added to the HUVECs showed markedly decreased VCAM-1 mRNA compared to 1 mM in the LPS group. In addition, the VCAM-1 mRNA was effectively reduced by the additional magnesium in the stimulated HUVECs relative to 1 mM in the LPS treatment group. HUVECs that were not stimulated and not blocked had a significant drop in VCAM-1 mRNA when cultured in 0.1 mM and 1 mM compared to the same magnesium concentration in the control group. However, VCAM-1 mRNA was markedly increased in the stimulated HUVECs that were grown in 0.1 mM $MgSO_4$ compared to stimulated HUVECs in 1 mM (Figure 5.15 A).

LPS/blocking TLR-4 and TLR-2 receptors and magnesium concentration interaction plays a role in the expression of IL-8 mRNA (P<0.001). When HUVECs were cultured in a low magnesium concentration and stimulated, IL-8 mRNA was considerably increased compared to cells cultured in the 1 mM magnesium undergoing the same treatment. However, after blocking the TLR-4 receptor, the mRNA expression of IL-8 in low magnesium HUVECs was lower, than in the unblocked LPS group. Similarly, after blocking the TLR-4 receptor in cells cultured in 1 mM $MgSO_4$, IL-8 mRNA decreased compared to the control group. Further, blocking the TLR-2 and TLR-4 receptors in stimulated cells grown in 5 mM $MgSO_4$, significantly reduced IL-8 mRNA relative to 1 mM $MgSO_4$ in the same treatment groups. Anti-TLR-4 also decreased IL-8 mRNA in the high magnesium cells. The high concentration of magnesium significantly reduced IL-8 mRNA in stimulated HUVECs compared to 1 mM in the same LPS group. Although, stimulated cells cultured in 0.1 mM $MgSO_4$ and blocked with anti-TLR-2 and anti-TLR-4 had decreased IL-8 mRNA

compared to 0.1 mM in control group, IL-8 mRNA in these groups was still higher than its expression in the same physiological concentration in the same treatment groups. It is worth mentioning that the HUVECs that were not LPS stimulated had low IL-8 mRNA concentrations compared to stimulated cells at the same magnesium concentration (Figure 5.15 B).

A



B

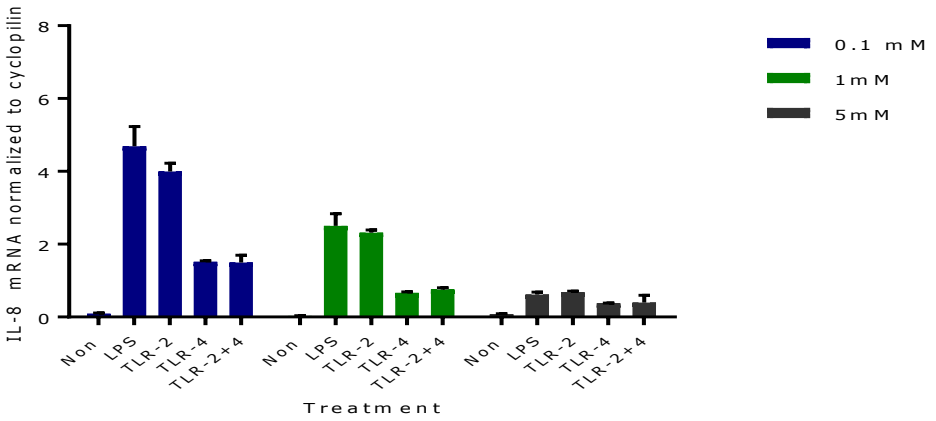
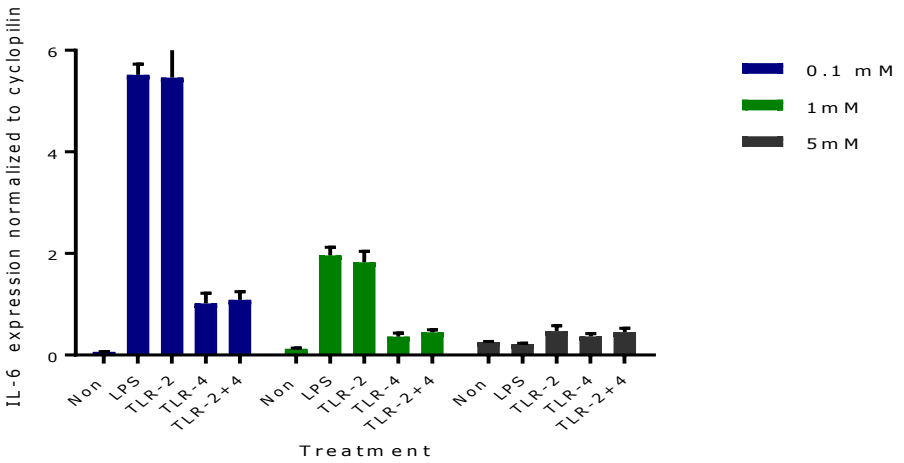


Figure 5.15 Effect of different concentrations of magnesium on HUVECs gene expression when treated or not treated with LPS and TLR antibodies. Confluent HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for three days, and then, TLR-2 and TLR-4 blocking antibodies (10µg/mL) were added to the HUVECs for 30 minutes before being stimulated, with or without LPS (0.5 µg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean ±SEM for n=4. A: Expression of VCAM-1 mRNA. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker ($P< 0.001$). B: Expression of IL-8 mRNA. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker ($P< 0.001$).

As shown in Figure 5.16A LPS treatment had a strong effect on IL-6 expression. It was also observed that the magnesium and LPS/blocker had a significant interaction effect (P<0.001) on the expression of IL-6 in the HUVECs. IL-6 mRNA decreased in the high magnesium concentration compared to the 1 mM $MgSO_4$ equivalent treatment group. In contrast, a lower magnesium concentration greatly increased IL-6 mRNA compared to 1 mM in the same LPS group. Blockade of TLR-4 receptors notably reduced IL-6 mRNA in LPS-stimulated HUVECs that were cultured in 0.1 mM $MgSO_4$ relative to the same magnesium concentration in the control group. In addition, blocking stimulated cells cultured in 1 mM $MgSO_4$ with anti-TLR-4 markedly decreased IL-6 mRNA compared to 1 mM in the control group. It is noted that, the HUVECs that were not blocked or stimulated had very low IL-6 mRNA concentrations compared to the same magnesium concentration in the control group.

MCP-1 mRNA was affected by LPS/blocking TLR-2 and TLR-4 receptors and magnesium concentration interaction (P= 0.005). Blocking stimulated HUVECs that were cultured in 1 mM $MgSO_4$ with anti-TLR-4 and anti-TLR-2 + TLR-4 significantly decreased MCP-1 mRNA compared to 1 mM in the control group. Moreover, the expression of MCP-1 markedly declined in the LPS-stimulated HUVECs that were grown in 0.1 mM $MgSO_4$ after inhibition by anti-TLR-4 antibodies compared to the same concentration in the control group. In addition, stimulated cells in 5 mM $MgSO_4$ showed significantly decreased MCP-1 mRNA concentrations compared to 1 mM in the same group. The MCP-1 mRNA concentration was much lower in the unblocked and unstimulated cells that were cultured in 0.1 mM and 1 mM compared to the same concentration in the control group (Figure 5.16 B). Table 5.2 shows a summary of the effect of blocking TLR-2 and TLR-4 on mRNA for genes associated with inflammation.

A



B

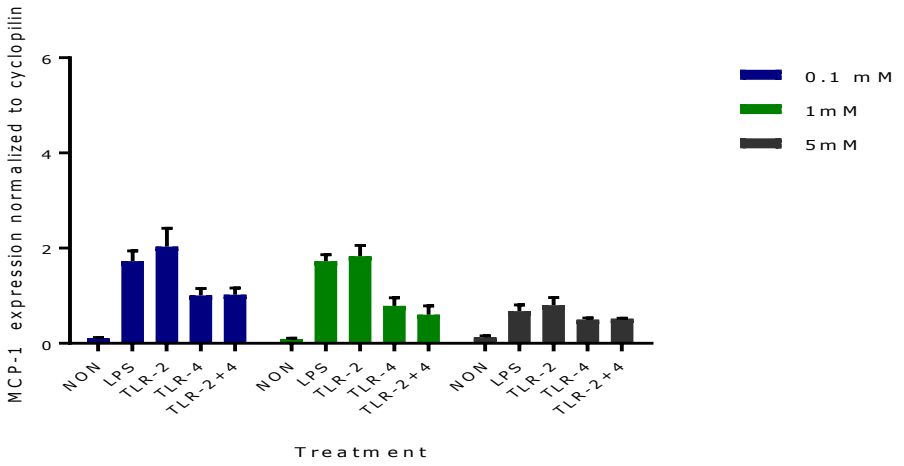


Figure 5.16 Effect of different concentrations of magnesium on HUVECs gene expression when treated or not treated with LPS and TLR antibodies. Confluent HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for three days, and then, TLR-2 and TLR-4 blocking antibodies (10µg/ mL) were added to the HUVECs for 30 minutes before being stimulated, with or without LPS (0.5 µg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean ±SEM for n=4. A: Expression of IL-6 mRNA. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker (P< 0.001). B: Expression of MCP-1 mRNA. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker (P= 0.005).

Table 5.2 Summary of the effect of blocking TLR-2 and TLR-4 on expression of mRNA for genes associated with inflammation

Genes	TLR2 blockade			TLR4 blockade			TLR2+4 blockade		
	0.1	**1**	**5**	**0.1**	**1**	**5**	**0.1**	**1**	**5**
NF-κB	↓	↓	-	↓	↓	-	↓	↓	-
ICAM-1	↓	-	-	↓	↓	↓	↓	↓	-
VCAM-1	↓	-	-	↓	↓	-	↓	↓	-
IL-8	-	-	-	↓	↓	↓	↓	↓	↓
IL-6	-	-	-	↓	↓	-	↓	↓	-
MCP-1	-	-	-	↓	↓	-	↓	↓	-

5.7.4.2 Effect of TLR-2 and TLR-4 Blocking on Adhesion Molecule and Cytokine Protein Concentrations

To determine the influence of TLR-2 and TLR-4 in the production of cytokines and adhesion molecules at a more functional level, we next measured IL-8 and VCAM-1 protein in the culture media by ELISA (experiment 8). HUVECs were cultured as described for the gene expression studies and then blocked with anti-TLR-2 and TLR-4 antibodies. The media was then collected after LPS stimulation.

Protein expression of IL-8 was significantly increased by LPS treatment. The results, as shown in Figure 5.17 A, demonstrated that magnesium concentration and LPS/blocker had a significant interaction effect ($P< 0.001$) on the protein concentration of IL-8. Insufficiency magnesium considerably increased secretion of IL-8 by stimulated HUVECs, compared to 1 mM $MgSO_4$ in the same treatment groups. In addition, the blocking HUVECs with anti-TLR-2 and anti-TLR-4 antibodies decreased IL-8 protein markedly compared to the control.

Figure 5.17 B shows that there was no significant interaction between magnesium concentrations and LPS/blocker on VCAM-1 protein concentration. There was a significant effect of magnesium concentration ($P< 0.001$), and the blocker ($P< 0.001$). Similar trends were observed to those shown for VCAM-1 mRNA (Figure 5.14A).

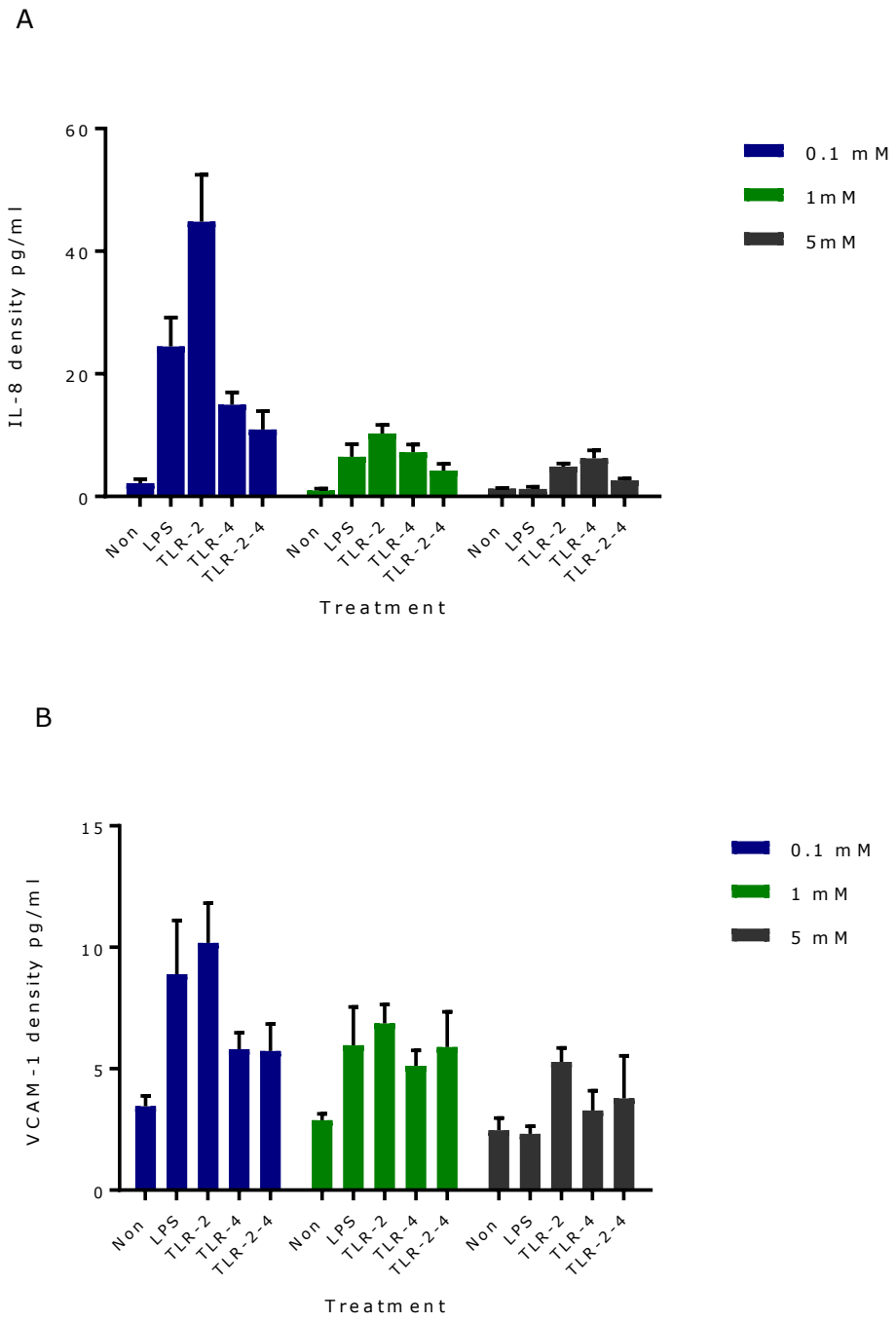


Figure 5.17 Effect of different concentrations of magnesium on HUVECs protein concentration treated or not with LPS and TLR antibodies. Confluent HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for three days, and then, TLR-2 and TLR-4 blocking antibodies (10µg/ mL) were added to the HUVECs for 30 minutes before being stimulated, with or without LPS (0.5 µg/ml) for 4 hours. culture media were collected and measured for VCAM-1 production by ELISA. Data are shown as mean ±SEM for n=4. A: Protein concentration of IL-8. Two-way ANOVA showed a significant interaction of magnesium × LPS/blocker ($P< 0.001$). B: Protein concentration of VCAM-1, Two-way ANOVA showed no significant interaction of magnesium × LPS/blocker ($P= 0.25$). There was a significant effect of magnesium concentration ($P< 0.001$), and blocker ($P< 0.001$).

5.8 Discussion

The key findings of this chapter were:

1. Low levels of magnesium enhance the expression of components of the NF-kB pathway, particularly in the presence of LPS. High magnesium concentrations suppress this pathway.
2. Low magnesium produces an inflammatory state in HUVECs and increases their response to LPS. This is, in part, explained by changes in expression of toll-like receptors.
3. Low levels of magnesium increase the release of the adhesion molecules ICAM-1 and VCAM-1.
4. Low levels of magnesium are associated with an increase in many inflammatory cytokines.
5. Blocking the TLR4 receptor inhibits NF-κB activation by LPS.

5.8.1 Effect of Varying Magnesium Concentrations on the NF-κB Pathway

Inflammation is a risk factor for the progression of atherosclerosis and can be mediated by NF-κB activation. NF-κB activation can be triggered by a number of factors such as UV radiation, reactive oxygen species, and bacterial or viral products such as LPS (Valen et al., 2001). Endothelial dysfunction is a major cause of inflammation resulting in atherosclerosis (Maier, 2012). Binding of LPS to the endothelial cell membrane receptors TLR2 and TLR4, activates the downstream signal for these receptors via the MyD88 Toll-domain containing protein, which phosphorylates IRAK1, which in turn activate TRAF6 (Zen et al., 1999). This upstream activation results in release of NF-κB from the IkB cytoplasmic inhibitor, followed by transfer to the DNA (Sun and Andersson, 2002). In the nucleus, NF-κB binds to the promoter region in the k-light chain (O'Neill and Kaltschmidt, 1997). Several studies have confirmed that NF-κB activation leads to increased transcriptional activity of many inflammatory genes involved in the development of atherosclerotic lesions (Manna and Ramesh, 2005, Tak and Firestein, 2001).

The NF-κB family consists of many members, including NF-κB 1 (p50), NF-κB 2 (p52), RelA (p65), RelB, and RelC. In the cytoplasm, NF-κB family members are bound to members of the IkB family, which includes six proteins (Savinova et al., 2009, Valen et

al., 2001). In unstimulated cells, IkB is an inhibitor protein that binds to NF-κB and keeps it inactive in the cytoplasm, preventing it from binding to the DNA (Berg and Baldwin, 1993). Translocation of NF-κB to the nucleus occurs when IkB is degraded upon phosphorylation by the upstream kinase IKK complex (Perrot-Applanat et al., 2011).

Magnesium deficiency affects endothelial cell function via the activator NF-κB, which contributes to atherosclerosis, which in turn leads to cardiovascular disease (Ferrè et al., 2010). This study aimed to investigate the effect of magnesium on the NF-κB pathway in basal conditions and in the presence of LPS.

As hypothesized, the present study confirmed that HUVECs cultured in low magnesium medium significantly increased NF-κB2 mRNA, as measured by qRT-PCR. In addition, magnesium treated (5 mM) HUVECs showed reduced expression of NF-κB2. As noted by Perrot-Applanat et al. (2011), TNF stimulation of HUVECs upregulated NF-κB1, NF-κB2 and IKB. Rochelson et al. (2007) found that after stimulation with 100 ng/mL of LPS, HUVECs treated with 5 mM of $MgSO_4$, exhibited an anti-inflammatory effect caused by magnesium-mediated inhibition of NF-κB translocation. Furthermore, a study that examined atherosclerotic tissue, using immunofluorescence confirmed the presence of active NF-κB in atherosclerotic lesions (Brand et al., 1996). Ferrè et al. (2010) confirmed that HUVECs cultured in the presence of a low magnesium concentration of 0.1 mM enhanced NF-κB activity in nuclear extract measured by electrophoretic mobility shift assay (EMSA), which further increased following treatment with 1 µg/mL of LPS. Moreover, Munoz et al. (1996) extracted the nuclei of HUVECs activated with TNF and assayed them using EMSA. The results showed increased binding of NF-κB to DNA in stimulated cells compared with unstimulated ones. Many reports have confirmed the activation of NF-κB result in increased IkB gene expressed (Sugimoto et al., 2012, Iwai et al., 2005). Moreover, increased IkB gene expression results in the activation of NF-κB, and the pattern of IkB expression often follow the gene expression of NF-κB (Lee et al., 2003).

The TLR family plays a role in identifying pathogens and exciting the innate immune response. TLR4 is activated in response to LPS and leads to activation of the NF-κB

pathway (Chong et al., 2004, Zhang and Ghosh, 2001). TLR2 can also recognize LPS and respond to it (Peters et al., 2003). The results of the present study confirmed the expression of TLR2 and TLR4 in LPS-stimulated HUVECs. In addition, HUVECs cultured with low magnesium showed a significant increase in expression of TLR4 but not TLR2. This is compatible with the results of Faure et al. (2001) and Zen et al. (1999), who showed that endothelial cells expressed TLR2 and TLR4 to activate NF-κB in response to LPS exposure. Many researchers have noted that LPS activates NF-κB in HUVECs via the membrane receptor TLR by MyD88 and Ikkβ (He et al., 2013, Andreakos et al., 2004). Similarly, Wang et al. (2011) found that TLR4, MyD88, and TRAF gene expression were upregulated after HUVECs were treated with 100 ng/mL of LPS. However, the present study found no difference in the expression of MyD88, TRAF, Ikk, and IkB proteins in the NF-κB pathway in response to treatment with magnesium. This suggests that other signalling pathways may be involved in the activation of NF-κB such as mitogen-activated protein (MAP) kinase pathway and GEF-H1-RhoA pathway, and must explain observed differences in inflammatory response with varying magnesium concentrations in culture.

Yang et al. (2007) demonstrated that, in the presence of LPS, TLR4 expression results in NF-κB activation via the MAP kinase pathway. The MAPK signalling pathway, which includes P38 MAPK and ERK1/2, induces NF-κB expression in response to LPS (He et al., 2013). Blocking ERK and P38 in HUVECs, stimulated with LPS, leads to decreased expression of NF-κB, suggesting that NF-κB translocation occurs via the MAPK pathway (Liu et al., 2011, Li et al., 2009). TLR signalling in LPS-activated HUVECs is regulated by the GEF-H1-RhoA pathway (Guo et al., 2012). Similarly, many reports have indicated that the Rho pathway plays a role in the NF-κB signalling pathway (Schraufstatter et al., 2001, Hippenstiel et al., 2000). In fact, the signalling pathway of LPS to induce cytokine secretion in endothelial cells is not well understood (Guo et al., 2012, Nakamura et al., 2008, Zeuke et al., 2002, Hippenstiel et al., 2000) LPS can initiate two pathways that result in cytokine expression, namely, the P38 MAPK and Rho pathways (Hippenstiel et al., 2000). Similarly, exposure of HUVECs to LPS results in NF-κB activation via the TLR4 receptor, which then

activates the Rho pathway, and MyD88 does not affect this pathway (Guo et al., 2012). The possibility that magnesium alters these pathways was not explored in the current work and this should be a priority for further study.

5.8.2 Effect of Different Concentrations of Magnesium on Adhesion Molecules

The immunoglobulin superfamily (IgSF) is one of the major adhesion molecule families, and is comprised of cell surface molecular that play essential roles in the intercellular adhesion that results in cell activation. Within this family, ICAM-1 and VCAM-1 are important members (Anderson and Siahaan, 2003, Teixeira, 1999). In fact, VCAM-1 is instrumental in increasing the adhesion of monocytes to the endothelial cell surface, which is the earliest step in the development of atherosclerosis (Ley and Huo, 2001). With an inflammatory stimulus, endothelial cells produce the intercellular adhesion molecules, ICAM-1 and VCAM-1, which enable ligand adhesion to leukocytes enabling them to transmigrate toward the inflammatory site (Perrot-Applanat et al., 2011, Hopkins et al., 2004, Munoz et al., 1996). The monocytes are then differentiated into macrophages as a part of the immune system, which then transform into foam cells through accumulation of oxidized LDL-cholesterol (Stannard et al., 2001).

Several studies have reported that stimulating endothelial cells with TNF results in the upregulation of VCAM-1 and ICAM-1 expression (Altannavch et al., 2004, Burne et al., 2001, McHale et al., 1999, Weber et al., 1994). Similarly, activating HUVECs with IL-4 results in an increased VCAM-1 expression that is dependent on activated NF-κB binding to the transcription site on the VCAM gene (Lee et al., 2001). It has also been confirmed that LPS-stimulated HUVECs express ICAM-1 and VCAM-1 via the TLR-4 signalling pathway, followed by the activation of the NF-κB pathway (Sawa et al., 2008). One study on animals conducted by Cybulsky et al. (2001) found that among mice expressing a low level of VCAM-1, there was a significantly reduced occurrence of early atherosclerosis lesions in the aorta compared to the control group. ICAM-1 has been found in the intima-media thickness and endothelium of rats with thromboangiitis obliterans (TAO, Buerger disease) suggesting a role in causing inflammation (Xu and Li, 2009). Additional research

has shown the presence of ICAM-1 in blood samples from cardiovascular disease patients (Lawson and Wolf, 2009, Vainer, 2005). Furthermore, patients who were considered to be relatively suffer from coronary artery disease had high levels of ICAM-1, and VCAM-1 related to the calcification of aortic stenosis (Cook-Mills et al., 2011). The findings of the present study confirm previous work, as HUVECs grown in 0.1 mM magnesium significantly increased the mRNA expression of NF-κB, and this increase was associated with increases in ICAM-1 and VCAM-1 mRNA. Furthermore, low magnesium concentrations promoted a sharp and significant increase in ICAM-1 and VCAM-1 gene expression in HUVECs stimulated with LPS. The same result was observed for ICAM-1 protein, which increased after being cultured in 0.1 mM magnesium.

Treating TNF-stimulated HUVECs with antioxidant herbs significantly decreased the adhesion of U937 monocytoid cells to the HUVECs, and reduced the expression of both ICAM-1 and VCAM-1 (Zhou et al., 2005). A further study in which HUVECs were stimulated with 0.1μg/ml LPS found that adding 10 mM $MgSO_4$ 30 minutes before adding LPS had a significant reductive effect on ICAM-1 protein expression (40% less than the control) measured by ELISA, and adding 10 mM $MgSO_4$ concurrently with the LPS lowered the ICAM-1 protein expression to 25% (Rochelson et al., 2007). Similarly, the current study has shown that culture HUVECs stimulated with LPS exposed to high concentrations of magnesium (5 mM) had significantly decreased gene expression for both ICAM-1 and VCAM-1.

In previous studies, the adhesion of monocytoid cells U937 to HUVECs stimulated with IL-1 markedly increased in low magnesium cultures (0.1 mM $MgSO_4$) (Ferrè et al., 2007). In contrast, the adhesion of the U937 cells to HUVECs stimulated with LPS (0.5 μg/ml) was decreased in a high magnesium culture (10 mM $MgSO_4$) (Maier et al., 2004a). In the same way, the present study highlighted that culturing HUVECs in low magnesium markedly enhanced the adhesion of THP-1 cells to HUVECs, and the 5 mM $MgSO_4$ treatment significantly hindered THP-1 binding. This confirms that magnesium effects on the expression of adhesion molecules in HUVECs has functional significance.

5.8.3 Effect of Different Concentration of Magnesium on Cytokines

The cytokines orchestrate the inflammatory response to infection and injury, and play an important role in vascular endothelial cell biology. IL-8 is a critical cytokine that attaches to neutrophils and moves them to the inflamed area. IL-8 expression depends on the activation of NF-κB (Guo et al., 2012, Hoffmann et al., 2002, Berg and Baldwin, 1993), and inflamed endothelial cells are the main producers of IL-8 (Li et al., 2005, DeForge and Remick, 1991). In addition, GRO and GROα work with IL-8 in attracting neutrophils (Baggiolini et al., 1995). MCP-1 cytokines also bind to mononuclear leukocytes (Lukacs et al., 1995). Moreover, IL-6 directs the generation of thrombocytes and the differentiation of B cells (Romano et al., 1997). IL-8 and MCP-1 have an essential role in the pathogenesis of arteriosclerosis and as such were of considerable interest in the current study.

As cytokines are considered as markers of events leading to atherosclerosis in *vivo*, we aimed in this chapter to examine whether different concentrations of magnesium can affect the expression of cytokines by HUVECs. By measuring the expression of mRNA and cellular proteins in HUVECs, we found evidence to support the hypothesis that different magnesium concentrations can alter the production of inflammatory cytokines. The results of this study showed that culturing stimulated HUVECs in low magnesium concentration (0.1 mM) rapidly increased the gene and protein expression of IL-8 and MCP-1 relative to the physiological concentration of magnesium 1 mM. Low magnesium concentration also significantly increased the protein expression of GRO, GROα, IL-2 and IL-3. These findings are consistent with the idea that magnesium deficiency increases the inflammatory response to LPS. As these pathways are dependent on NF-κB activation the findings fit well with data from earlier in this chapter (discussed in 5.8.1 and 5.8.2).

These results are supported by a previous study that found that low magnesium concentrations (0.1 mM) increased the protein expression of IL-8 in HUVECs, but did not affect the protein expression of TNF-α (Ferrè et al., 2010). The effects of LPS treatment noted in this study are also corroborated by other studies, such as Hwang et al. (2013)

which found that treating HUVECs with LPS 200 ng/ml for 15 minutes significantly raised the gene expression of MCP-1. Likewise, Sawa et al. (2008) found that stimulating HUVECs with 100ng/ml of LPS for 24 hours increased the gene expression of IL-8 and IL-6. Activated HUVECs with membrane attack complex (MAC) also release IL-6 and IL-8 proteins (Kilgore et al., 1996). Infected HUVECs with the gram-negative, intracellular bacterium Rickettsia conorii significantly increased the protein secretion of IL-6 and IL-8 (Kaplanski et al., 1995). HUVECs that were stimulated with TNFα resulted in the overexpression of IL-6, IL-8, MCP-1 and GOR mRNA (Perrot-Applanat et al., 2011). To the best of our knowledge no other studies examining the effect of low magnesium on the protein expression of IL-6, MCP-1, GOR, GROα, IL-2 and IL-3 in HUVECs have been found in the published literature. Also, there has been no report of the effect of high magnesium concentration in decreasing the protein concentrations of IL-6, IL-2 and MCP-1 in HUVECs. The current study also found that a high concentration of magnesium significantly downregulated IL-6 and MCP-1 mRNA, and decreased the concentration of IL2 and IL-6 proteins in HUVECs. This result is consistent with the study of Tam et al. (2011), which found that treating pregnant rats with LPS significantly increased the release of IL-6, MCP-1 and GRO in to the serum compared to the control. Treating these rats with magnesium before and after LPS treatment produced a marked decrease in levels of IL-6, MCP-1 and GRO in the serum. Likewise, in response to LPS, inflamed endothelial cells increase gene production of cytokines, such as IL6 and IL8, via to NF-κB activation. Antioxidant treatment can inhibit HUVECs from expressing cytokines as well (Munoz et al., 1996).

The findings of this study indicated that magnesium concentration did not have any significant effect on the protein expression of TNFβ, IL-1, IL4, IL5, IL7, IL10, IL12, IL-13 and IL15. Nilsen et al. (1998) also found that stimulating HUVECs with TNF-α did not change the mRNA expression of IL-7, and the mRNA expression of IL-4, IL5, IL-10, IL 12 and IL-13 was not detectable. Additionally, when HUVECs were stimulated with LPS, the mRNA expression of IL-1B was unaffected. In contrast, Brand (1996) indicated that the

upregulation of NF-κB protein increased the gene expression of TNF-α and IL-1B. We hypothesize that magnesium did not affect the pathway that activates these cytokines.

5.8.4 Contribution of TLR-2 and TLR-4 in Lipopolysaccharide (LPS) Signalling in HUVECs

As discussed above, this study showed that HUVECs express TLR-2 and TLR-4, and we demonstrated that LPS stimulation (presumed to be through TLR-4) results in the activation of NF-κB gene expression, which leads to upregulation of expression of adhesion molecules and inflammatory cytokines genes in HUVECs. In addition, the study demonstrated that a low concentration of magnesium enhanced this inflammatory activation. To confirm the role that magnesium plays in inhibiting the LPS-TLR signalling pathway, and to identify the function of the TLR-2 and TLR-4 receptors in activating NF-κB in response to LPS, the effects of TLR-2 and TLR-4 receptor blockade by neutralising antibodies was evaluated in different magnesium concentrations.

Most current research focuses on the role of TLR4 in eliciting the response of HUVECs to LPS. TLR2 has also been implicated in the LPS pathway, but no study has previously investigated the effect of blocking TLR2 on NF-κB activation and the release of cytokines in HUVECs. Our results confirm the hypothesis that TLR2 and TLR4 both play a role in regulation of NF-κB activation as blocking these receptors significantly inhibited the effect of LPS on NF-κB gene expression in the HUVECs cultured in a low magnesium concentration. This showed that magnesium deficiency can influence processes which depend on both receptors. These results are consistent with those reported by Guo et al. (2012), which indicated that blocking TLR-4 in HUVECs inhibits the effect of LPS in activating NF-κB. Similarly, blocking TLR-4 in human dermal microvascular endothelial cells (HMEC) stimulated with LPS results in inhibition of NF-κB activity (Faure et al., 2000).

Interestingly, the high magnesium concentration was shown to inhibit LPS activation of NF-κB more efficiently than anti-TLR-2 and anti-TLR-4 antibodies. Magnesium appears to

have a direct impact the TLR-4 receptors, based on the fact that magnesium does not modify the expression of MyD88 and, subsequently, TRAF6. The anti-inflammatory impact of magnesium in the TLR-LPS pathway may occur via several mechanisms:

1) Magnesium can directly inhibit the LPS induced expression of the TLR-4 receptor (as confirmed in this study) and therefore limit NF-κB activation.

2) Action of magnesium as a calcium channel blocker and effect on the intracellular calcium concentration. Calcium is a major promoter of inflammation (Kupetsky-Rincon and Uitto, 2012, Mazur et al., 2007) due to its role in the activation of the TLR-4 receptors (Chiang et al., 2012).

3) Impact of high magnesium on blocking the NMDA receptors, which regulate the pre-inflammatory response (Liu et al., 2013, Weglicki et al., 2010). Such blocking leads to reduced activity of the TLR-4 receptor, and magnesium has an antagonistic effect on the NMDA receptor (Chen et al., 2010). Mg^{2+} can suppress NMDA receptors through a voltage dependent manner (Weglicki et al., 2010).

Another substantial finding of this study is that a high magnesium concentration significantly inhibits the expression of the adhesion molecules; ICAM-1 and VCAM-1 in stimulated HUVECs. The highest magnesium concentration facilitated the inhibition of expression of ICAM-1 and VCAM-1. TLR-2 and TLR-4 play a significant role in ICAM-1 and VCAM-1 release, and the blocking of these receptors also significantly downregulated the expression of ICAM-1 and VCAM-1 when cells were exposed to a low magnesium concentration. The results of the present study agree with the findings reported by (Nakamura et al., 2008) who found that incubating stimulated HUVECs with anti-TLR-2 and anti-TLR-4 antibodies significantly inhibited the mononuclear cell adhesion to the HUVEC's surface.

One of the main findings of this study was that inhibition of TLR-4 by neutralising antibodies in stimulated HUVECs, cultured in a low magnesium concentration, decreased the LPS induced expression of the inflammatory cytokines IL-8, IL-6 and MCP-1 mRNA.

Moreover, this inhibitory effect of the antibodies was enhanced with exposure to a high magnesium concentration. Furthermore, the addition of magnesium significantly inhibited the release of IL-8, IL-6 and MCP-1 in cells following LPS treatment. These results are consistent with the findings reported by Hippenstiel et al. (2000), who clarified that activated NF-κB was important in upregulating IL-8 expression and that IL-8 expression was inhibited in LPS-stimulated HUVECs when Rho was blocked. The work of Masood et al. (2001) also indicated that, after stimulating HUVECs with the serum of patients with bacterial infections and incubating them with TLR antibodies, the blocking with anti-TLR-4 markedly decreased the expression of IL-8 by 40%, whereas the blocking with anti-TLR-2 only slightly decreased the expression of IL-8. The present study similarly found that the effects of TLR-4 blockade were greater than seen with TLR-2 blockade. Shimamoto et al. (2006) observed that the TLR-4 mutation in mice inhibited the expression of IL-6 and MCP-1 mRNA in the animals' hearts. Furthermore, incubating HUVECs with siRNA to silence TLR-4 for 24 hours, then stimulating them with LPS significantly decreased the expression of MCP-1 (Ishikado et al., 2009). To the best of our knowledge, this is the first study to compare the effects of TLR-2 and TLR-4 receptor blockade in varying magnesium concentrations.

5.9 Limitations

The relationship between mRNA and protein concentrations need to be addressed. This may be related to simple analysis numbers which had low numbers for some assays and that may underpowered on those analyses, or less sensitivity of the array compared to the qRT-PCR. There was a weak correlation between mRNA and the level of protein (Koussounadis et al., 2015). This may be due to mRNA degradation, which affects protein translation (Greenbaum et al., 2003). Maier et al. (2009) reported that many technical and biological factors affect the correlation between mRNAs and proteins, the most important factor being the half-life of the protein. Moreover, the protein activity can be influenced by phosphorylation and degradation (de Sousa Abreu et al., 2009), and the activated protein acts quickly so it may be degraded very rapidly (Vogel and Marcotte, 2012).

5.10 Conclusion

In summary, these data show that magnesium concentration is inversely associated with the expression of NF-κB mRNA. We also show, for the first time, that low magnesium concentrations and LPS stimulation increase adhesion molecule expression, which induces an inflammatory phenotype in endothelial cells. The main finding of this study is that low magnesium concentrations promote inflammatory cytokine expression in the presence of LPS, which has been connected to the pathogenesis of many cardiovascular diseases. The influence of magnesium in modulating release of pro-inflammatory proteins occurs through the TLR4 receptor. Conversely, additional magnesium suppresses the inflammatory response through down regulation of TLR4 receptors.

In conclusion, magnesium plays an important role in regulating the inflammatory status of endothelial cells through regulation of the TLR-LPS pathway. Based on these in *vitro* findings, we suggest that in *vivo* consumption of magnesium, from natural resources or oral magnesium supplementation, could have an anti-inflammatory effect on endothelial cells and potentially protect against cardiovascular diseases. Additional studies are needed to more accurately clarify the mechanism through which magnesium modulates the LPS-TLR pathway in HUVECs. By increasing understanding of these processes, the role of magnesium nutrition in determining risk of cardiovascular disease and the potential role of this mineral in public health will be more fully elucidated.

6

General Conclusions and Future Work

6 General Conclusions and Future Work

This chapter aims to present summaries and conclusions for all findings that were obtained from the experimental work previously discussed in this thesis.

6.1 Summary

Magnesium is an essential ion in cells. Influencing both physiological and biochemical processes, it is also required as a substrate for the activation of various enzymes (Saris et al., 2000). Magnesium additionally influences carbohydrate, protein and ATP synthesis, which will have an effect on cell proliferation and broader functions (Banai et al., 1990). Magnesium is a Ca^{2+} channel blocker and has anti-inflammatory activity (De Baaij et al., 2015). Low magnesium results in increased inflammatory responses (Rochelson et al., 2007) associated with atherosclerosis (Mazur et al., 2007). Low magnesium also contributes to numerous cardiovascular risk factors, such as hyperlipidaemia, hypertension and diabetes (Ferrè et al., 2010).

HUVEC cells were used in this *in vitro* study to expand our understanding of the influence of different concentrations of magnesium on the behaviour of HUVECs as well as how endothelial dysfunction resulting from low magnesium may alter cell proliferation, promote NF-κB activation, adhesion molecules, cytokine production and some of the key genes involved in endothelial cell functions. The study was carried further using a microarray analysis to examine gene expression profiles to obtain further information regarding the impact of magnesium on HUVECs.

The overall objectives of the project were achieved first by measuring the proliferation of HUVECs with different assays to examine the effect of magnesium concentration on cell proliferation and evaluate the protective ability of magnesium in counteracting the effects of LPS-induced cell death. Then the relevant genes, pathways and regulatory networks that depend on magnesium concentration in HUVECs were identified using microarray analysis. The final objective was to investigate the impact of magnesium on endothelial cell function and their responses to inflammatory signals by using qRT-PCR and other

assays to measure the concentrations of adhesion molecules, cytokines and proteins in the NF-κB pathway.

6.2 Summary of Novel Findings from This Thesis

In conclusion, for the first time, this study has shown the following:

1. High magnesium concentration protects HUVECs from LPS-induced cell death.
2. Magnesium influences the expression of 12.3% of the HUVECs transcriptome, as indicated by a microarray analysis for HUVECs treated with different concentrations of magnesium.
3. A high magnesium concentration decreases the expression of MCP-1 and IL-6 mRNA in LPS-stimulated HUVECs.
4. A high magnesium concentration decreases the intracellular protein concentration of IL-2 and IL-6.
5. A high magnesium concentration reduces the LPS induced IL-8 mRNA.
6. A low magnesium concentration increases TLR4 mRNA expression in LPS-stimulated HUVECs.
7. LPS-stimulated HUVECs that are cultured in low magnesium increased the LPS induced upregulate of the expression of ICAM-1 and VCAM-1 mRNA.
8. A low magnesium concentration increases both the MCP-1 mRNA expression and the intracellular protein concentration in LPS-stimulated HUVECs.
9. Low magnesium increases the intracellular protein concentration of GRO, GROα, IL-2, IL-3 and IL-8.
10. Blocking the TLR4 receptor inhibits the NF-κB activation by LPS in HUVECs cultured in a low magnesium concentration.
11. Blocking the TLR2+4 receptors in HUVECs inhibits the LPS-induced expression of ICAM-1, VCAM-1, IL-8, MCP-1 and IL-6 mRNA.

Figure 6.1 summarises the outcomes of the experiments reported in this thesis and shows their relation to pre-existing knowledge of the effects of magnesium on vascular

endothelial cell function. Overall the findings indicate that low magnesium status has a pro-inflammatory effect in HUVECs, with increased expression of inflammatory cytokines, which if extrapolated to the whole body could promote atherosclerosis. High magnesium in general has the opposite effect of low magnesium, suggesting that the relationship between magnesium and cardiovascular risk factors is dose-dependent.

Novel finding	What was known	Impact on disease
Growth / proliferation		
High magnesium concentration protected the HUVECs from LPS-induced cell death. Magnesium concentrations alter 81 genes, related to cell proliferation, and 20 genes involved in cell death.	Low magnesium levels can decrease endothelial cell proliferation (Ferrè et al., 2007, Maier et al., 2004b, Banai et al., 1990).	Vascular injuries contribute to thrombosis and atherosclerosis.
Magnesium and HUVECs function		
Magnesium induced a 12.3% change in HUVECs global gene expression. 581 genes were up- or downregulated in both low- and high-magnesium conditions.	Mg^{2+} is also necessary for the regulation of genetic stability (De Baaij et al., 2015, Anastassopoulou and Theophanides, 2002, Hartwig, 2001).	Endothelial function
820 genes respond to stress, 61 of which are involved in chemokine and cytokine signalling pathways	Magnesium plays a role in the regulation of inflammation (Sugimoto et al., 2012, Mazur et al., 2007, Bernardini et al., 2005).	
	Low magnesium concentrations significantly increased the mRNA expression of TRPM-7 (Baldoli et al., 2013).	
No significant variations in ET-1 mRNA between different magnesium concentrations.	Magnesium reduces the gene expression of ET-1 in the aorta and heart (Berthon et al., 2003). Decreased levels of plasma ET-1 (Serebruany et al., 1997)	Decrease in blood pressure

Varying the magnesium concentrations did not significantly change eNOS mRNA, protein concentration or nitric oxide production in HUVECs.	High magnesium increased NOS activity in *vitro* (Maier et al., 2004a). Low magnesium concentration increased NOS activity in *vitro* (Bernardini et al., 2005). High magnesium concentrations (10 mM $MgCl_2$) significantly decreased nitric oxide release and NOS activity in *vitro* (Howard et al., 1995).	Reduction in eNOS increases the number of platelets and adhesion molecules, thrombogenicity and inflammation, which can, in combination, lead to cardiovascular events.
	Inflammation response	
Low magnesium concentration increases the TLR4 mRNA expression in LPS stimulated HUVECs.	Binding of LPS to the HUVECs membrane receptors TLR2 and TLR4, activates NF-κB (Zen et al., 1999). Low magnesium concentration of 0.1 mM enhanced NF-κB activity (Ferrè et al., 2010). High magnesium inhibition of NF-κB translocation (Rochelson et al., 2007).	NF-κB activation leads to increased transcriptional activity of many inflammatory genes involved in the development of atherosclerotic lesions.
Low magnesium upregulate the expression of ICAM-1 and VCAM-1 mRNA in LPS stimulated HUVECs. Culturing HUVECs in low magnesium markedly enhanced the adhesion of THP-1 cells to HUVECs, and the 5 mM $MgSO_4$ treatment significantly hindered THP-1 binding.	Stimulated HUVECs with LPS activation NF-κB expression which increase the expression of ICAM-1 and VCAM-1 (Sawa, 2008).	Increasing the adhesion of monocytes to the endothelial cell surface, which is the earliest step in the development of atherosclerosis.

Low magnesium increases the intracellular protein concentration of GRO, GROα, IL-2, IL-3 and IL-8 in LPS stimulated HUVECs. High magnesium concentration decreases the intracellular protein concentration of IL-2 and IL-6. High magnesium concentration decreases the expression of IL-6, and MCP-1 mRNA in LPS stimulated HUVECs. ---------- Blocking the TLR4 receptor inhibits the NF-κB activation by LPS in HUVECs cultured in a low magnesium concentration Blocking the TLR2+4 receptors in HUVECs inhibits the LPS induced expression of ICAM-1, VCAM-1, IL-8, MCP-1 and IL-6 mRNA High magnesium concentration reduced the LPS induced IL-8 mRNA	Low magnesium concentrations (0.1 mM) increased the expression of IL-8 in HUVECs (Ferrè et al., 2010). Treating rats with magnesium before and after LPS treatment produced a marked decrease in level of IL-6, MCP-1 and GRO in the serum (Tam et al., 2011). ---------- Blocking TLR4 in HUVECs inhibits the effect of LPS in activating NF-κB (Guo et al., 2012). Incubating HUVECs with siRNA to silence TLR4 for 24 hours, then stimulating them with LPS significantly decreased the expression of MCP-1 (Ishikado et al., 2009). Incubating LPS stimulated HUVECs with anti-TLR2 and anti-TLR4 antibodies significantly inhibited the mononuclear cell adhesion to the HUVEC's surface (Nakamura et al., 2008). Blocking with anti-TLR4 markedly decreased the expression of IL-8 (Masood et al., 2001)	Cytokines are considered markers of events leading to atherosclerosis. ---------- Regulating the inflammatory status of endothelial protect against cardiovascular diseases

Figure 6.1 Summary of experimental outcomes. Green arrows show the possible linkage of novel findings to disease impact. Red blocks highlight discord between the current study and the literature.

6.3 Effect of Magnesium on the Proliferation of HUVECs

The proliferation of HUVECs is an important step in vessel angiogenesis, not only during the embryonic period but also in mature vessels. HUVECs proliferation is crucial to healing any injured wound in the vascular wall and maintaining the regression vessels (Cines et al., 1998). A significant amount of evidence has confirmed that low magnesium inhibits the proliferation of HUVECs (Wolf and Cittadini, 1999, Banai et al., 1990). This results in impaired regrowth of endothelial cells, which allows vessel injuries that lead to atherosclerosis, an effect mediated by developing arterial thrombosis and increased proliferation of smooth muscle cells (Banai et al., 1990). The mechanism through which magnesium affects cell division appears to involve the magnesium requirement for protein and DNA synthesis and carbohydrate metabolism (Rubin, 2005). Magnesium ions are also required for the maintenance of DNA structure, replication and transcription of DNA and translation of RNA (Wolf and Cittadini, 1999).

The processes that regulate the cell cycle also appear to be magnesium sensitive. Cyclin-dependent kinases (CDKs) are proteins that control the cell cycle; the transition from the G1 to the S phases depends on CDK2, CDK4 and CDK6. These CDKs can be inhibited by WAF1/Cip1 cyclin-dependent kinase inhibitor families, which include p21, p27 and p57 (Ikari et al., 2011). Low magnesium increases the expression of the cell cycle inhibitors p27 and p21 and increases oxidative damage to DNA. DNA damage prevents cells from passing through cell cycle checkpoints and can result in cell cycle arrest or apoptosis (Wolf et al., 2008a).

Our *in vitro* experiments, presented in Chapter 3, demonstrated that magnesium had a significant effect on the DNA content of the HUVECs in both a concentration- and a time-dependent manner, a result which largely confirmed the findings of other studies that low magnesium limits HUVECs proliferation (Ferrè et al., 2007, Maier et al., 2004b, Banai et al., 1990). The results from Chapter 4 concur with the data obtained from the proliferation assays, which identified further mechanisms through which HUVECs proliferation may respond to magnesium. These effects are because magnesium influences expression of

many genes that regulate these processes. The Gene Ontology analysis for the HUVECs' microarray data showed for the first time, to the best of our knowledge, that a number of genes implicated in regulation of cell proliferation were sensitive to magnesium concentrations in culture medium. High and low magnesium concentrations altered 53 and 28 genes related to cell proliferation, respectively. For example, CDC28 protein kinase regulatory subunit 1B (CKS1B) is downregulated in low magnesium resulting in decreased proliferation. CKS1B regulated the degradation of the CDK inhibitor P27, which lead to cell proliferation (Shi et al., 2010, Zhan et al., 2007). In addition, proliferation-associated 2G4 (PA2G4), which is also known as ErbB3-binding protein 1 (EBP1), was found to be downregulated in low magnesium. PA2G4 is a protein that is involved in regulating cell proliferation and preventing cell apoptosis (Neilson et al., 2017, Figeac et al., 2014, Kowalinski et al., 2007).

The effect of magnesium on cell proliferation is therefore about much more than its role in making new DNA. Magnesium is also needed in ribosome activity which control the protein synthesis, which in turn controls DNA synthesis. Further, magnesium activates the growth factors that increase protein synthesis and glucose uptake such as epidermal growth factor (EGF) (De Baaij et al., 2015).

The present study showed a protective effect of magnesium on HUVECs against the effects of LPS-induced cell death. Moreover, the microarray results show that magnesium can alter the expression of 20 genes involved in cell death / apoptosis.

As magnesium contribution to HUVECs proliferation it may be inferred that it would enable healing of endothelial injury and to prevent thrombosis and atherosclerosis (Maier et al., 2004a, Banai et al., 1990).

6.4 Effect of Magnesium Concentration on mRNA Expression in HUVECs

To the best of our knowledge, this is the first study to present the global gene expression from a microarray analysis for HUVECs treated with different concentrations of magnesium. This study demonstrated that magnesium altered expression of up to 12.3% of the HUVECs genome; the high magnesium concentration altered the highest percentage

of the genes (6.5%), while the low concentration altered a smaller fraction of genes (5.8%). The mechanism behind the effect of magnesium on gene expression is not fully understood and some studies have investigated the mechanisms that can clarify how magnesium can impact the percentage of affected genes. Firstly magnesium is important to maintain the fidelity of DNA during the DNA replication as a substitute in DNA polymerase. Further, magnesium plays an essential role as a cofactor in DNA repair processes (Hartwig, 2001). Moreover, magnesium is important for RNA transcription due to its role as a cofactor of RNA polymerases (De Baaij et al., 2015). So, magnesium has a critical role in preserving genomic stability. On top of the magnesium requirement in maintaining DNA stabilization, it is also required for protecting DNA against oxidative damage. The positive charge of magnesium ions neutralizes the negative charge of phosphate groups to stabilize the double helix of the DNA. Neutralization of the negative charge of DNA protects it from the hydroxyl radical cleavage events that cause DNA damage (Anastassopoulou and Theophanides, 2002). Increasing the intracellular Ca^{2+} due to low extracellular magnesium can lead to impacts on the mitochondrial respiratory activity, resulting in increased release of the main sources of free radicals / ROS (Petrault et al., 2002). Increasing ROS causes DNA damage and affects the DNA repair mechanisms (Wolf and Trapani, 2008).

Magnesium can alter the expression of many transcription factors and so influence the expression of large networks of genes. When Ożgo et al. (2007) used a DNA array for 1176 genes to analyse mouse kidney gene expression, they found that most of the genes that responded to magnesium change belong to the transcription factor family. For instance, low magnesium can activate the expression of the transcription factor NF-κB which then promotes the expression of many inflammatory responses (Maier, 2012, Rochelson et al., 2007). Based on the results of this study, low magnesium increases the inflammatory responses by increasing the expression of transcription factor NF-κB, whereas high magnesium prevents NF-κB activation. And the result from Go analysis of molecular functions in chapter 4 found that the highest enrichment score was for

regulation of transcription factor activity when HUVECs were treated with low magnesium concentration.

MicroRNA (miRNA) modulation is another possible mechanism for magnesium action, due to the important role of magnesium in miRNA biogenesis and in cleavage of the target mRNA (Nam et al., 2014). This is because there are interactions between magnesium and RNA-induced silencing complex (RISC) protein, which is magnesium-dependent (Beckett et al., 2014). In the presence of magnesium, miRNA are incorporated into RISC to bind to the target mRNA sequence (Chamorro-Jorganes et al., 2013). RISC contains Dicer enzyme and some cofactors such as Argonaute2 (Ago2) (Staszel et al., 2011), which are important components in the RISC complex that identify the mRNA. Also, three magnesium ion binding sites are located at the small RNA binding domain of Ago2 (Ma et al., 2012). It is known miRNAs regulate gene expression and play a critical role in many pathological and physiological processes, by blocking mRNA translation or inducing mRNA degradation (Staszel et al., 2011).

The TRPM7 ion channel may also provide a mechanism through which magnesium can have widespread effects upon gene expression. TRPM7 contains a protein kinase and regulates magnesium homeostasis (Ryazanova et al., 2010). The serine/theronine kinase can give rise to fragments TRPM-7 cleaved kinases (M7CKs) that translocate to the nucleus and then alter the structure of chromatin, which would modify transcription of other genes (Krapivinsky et al., 2014). The current study found that changing magnesium concentration alters the expression of TRPM7 and so these effects on chromatin, although not explored, could partly explain some of the observed findings.

Magnesium can also affect DNA methylation (Takaya et al., 2011). One of the DNA methyltransferase (MTases) enzyme classes, Eco P15I DNA methyltransferase (Eco P15I DNA MTase), requires Mg^{2+} for methylation activity, suggesting that magnesium ions can enhance methylation (Bist and Rao, 2003). Eco P15I DNA MTase leads to methyl group transfer to DNA (Reddy and Rao, 1998). DNA methylation changes the gene expression

from changes in DNA structure rather than sequence, and this would suppress transcription (Crider et al., 2012).

The results reported in Chapter 4 showed that magnesium plays a role in many physiological functions and highlights the fact that changing magnesium concentration can influence a number of genes involved in many biological processes, molecular functions and cellular components according to the Gene Ontology analysis.

Interestingly, the Gene Ontology analysis showed that 820 genes relate to stress (Chapter 4), which is compatible with the results of Chapter 5, which focused on the anti-inflammatory role of magnesium. High magnesium influenced 33 of the genes involved in inflammation mediated by the chemokine and cytokine signalling pathways, and downregulation the mRNA expression for many of the interleukin genes, such as IL8, IL5, IL33, IL6R and the chemokine genes, such as CXCL1 (GROα) and CXCL2 (GROβ). Low magnesium altered expression of 28 genes involved in inflammation that is mediated by the chemokine and cytokine signalling pathways.

Following the microarray analysis, the study identified the effect of magnesium treatment on some candidate genes involved in HUVECs functions. The results found that low magnesium significantly increased TRPM-7 mRNA expression. In comparison, HUVECs treated with different magnesium concentrations had no effect on ET-1 or eNOS mRNA expression, eNOS protein concentration or nitrite levels in the culture media.

6.5 The Effect of Magnesium on the Inflammatory Response in HUVECs

Based on results which indicated that magnesium impacts on the expression of many genes involved in the inflammatory response (Chapter 4), this study examined the impact of different concentrations of magnesium on the NF-κB pathway, the adhesion molecules and the inflammatory cytokines in HUVECs. Numerous studies have clarified that magnesium deficiency activates leukocyte and macrophage and promotes the production of inflammatory cytokines and increases oxidative stress (Nielsen, 2010, Tam et al., 2003). In response to low magnesium, endothelial cells release adhesion molecules to bind leukocytes and express cytokines, and this activation occurs through the NF-κB pathway

(Rayssiguier et al., 2010). The activation of the inflammatory dependent response causes an endothelial dysfunction, which is the main cause of atherosclerosis (Maier, 2003), one of the cardiovascular disease risk factors (Hansson et al., 2006). It has been reported that the underlying mechanism is that magnesium is a calcium antagonist and low magnesium concentrations increase the level of intra-cellular calcium, which is the main promoter for NF-κB activity. Moreover, magnesium is an NMDA receptor blocker; the inhibition of this receptor inhibits NF-κB activity and pro-inflammatory release (Lee et al., 2015, Su et al., 2013, Rayssiguier et al., 2010, Lin et al., 2009). But this study hasn't test this mechanism, and it could be due to other factors as the study have described above- many ways that magnesium could influence expression.

The main finding from the current study is that magnesium may reduce the risk of inflammation and that magnesium deficiency may induces HUVECs inflammation. In Chapter 5, the results indicated that HUVECs express TLR2 and TLR4 receptors and that low magnesium upregulated the TLR4 mRNA expression in LPS-stimulated HUVECs. Therefore, LPS activated NF-κB2 mRNA expression in HUVECs cultured in low magnesium concentration; whereas, high magnesium concentrations decreased the NF-κB2 activation. Noticeably, magnesium did not affect the sequential recruitment in the NF-κB signalling pathway.

A low magnesium concentration increased the release of adhesion molecules ICAM-1 and VCAM-1, as well as the expression of many cytokines such as IL-8, MCP-1 IL-2, IL-3, IL-8, MCP-1, GRO and GROa. In contrast, expression was downregulated in high magnesium concentrations.

This study confirmed that the TLR-4 receptor is essential in LPS signalling to activate NF-κB. The results showed that blocking the TLR-4 receptor significantly decreased the LPS induced expression of NF-κB, ICAM-1, VCAM-1, IL-8, IL-6 and MCP-1. Importantly, the study clarified a novel finding of the role of magnesium in inhibiting the TLR-LPS signalling pathway to activate NF-κB and release adhesion molecules and cytokines in HUVECs.

Numerous mechanisms could explain the anti-inflammatory effects of magnesium on the TLR-LPS pathway, as discussed in Chapter 5.

6.6 Future Work

The findings from this thesis provide important results regarding the role of magnesium in HUVECs function. The *in vitro* experiments conducted in this study have provided a valuable platform for future investigation. Having identified that magnesium influences the expression of a high proportion of the genome in HUVECs, it would be of interest to conduct a further microarray analysis on HUVECs cultured in different magnesium concentrations, stimulated with LPS, with the subsequent results compared with those of previous experiments. The aim would be to identify a wide range of new pathways that contribute to HUVECs inflammation, the transcription factors that activate these pathways, as well the underlying mechanisms, such as the role of magnesium in altering calcium concentrations and the roll of calcium in cell signalling. Future work should also attempt to identify the TLR4–NF-κB pathway that the magnesium influences, again exploring how magnesium impacts the regulation of this system. Further, future work could examine whether a protein such as TRPM7 might play a central role in regulating HUVECs proliferation and expression.

Another major area of research would be the role of magnesium in the regulation of HUVECs' miRNA and how that affects normal endothelial function on preventing atherosclerosis. Future *in vivo* experimentation such as clinical studies that provide supplements to elderly patients would be beneficial in evaluating the effect of magnesium supplementation on inflammation, atherosclerosis and improvements in cardiovascular indicators such as cytokine profiles.

6.7 Conclusion

This study investigated the role of magnesium in regulating endothelial cell activity. Endothelial cells are gatekeepers between the blood and tissue; therefore, endothelial dysfunction is implicated in many vascular diseases. It is becoming clear that the inflammatory response plays a critical role in the pathogenesis of atherosclerosis, and it is

a significant aim for anti-atherosclerotic interventions. This thesis has confirmed that low magnesium promotes many inflammatory responses, thus leading to endothelial dysfunction. It could be suggested that magnesium deficiency could be a contributor to development of atherosclerosis. As poor magnesium status is generally unnoticed in the general population, greater attention to magnesium nutrition may be warranted.

In brief, to provide an overview of the anti-inflammatory role of magnesium in HUVECs, the results of our *in vitro* study showed that magnesium suppresses HUVECs' inflammatory responses induced by LPS regulation of the TLR4/ NF-κB pathway. A noteworthy outcome of this study was to provide evidence that treatment with magnesium can inhibit expression of certain adhesion molecules and cytokines. Based on the current evidence of the effect of magnesium on endothelial function, magnesium supplementation might be considered in patients with early-stage artery diseases to prevent the further development of blood vessel injury and atherosclerotic lesions.

7 References

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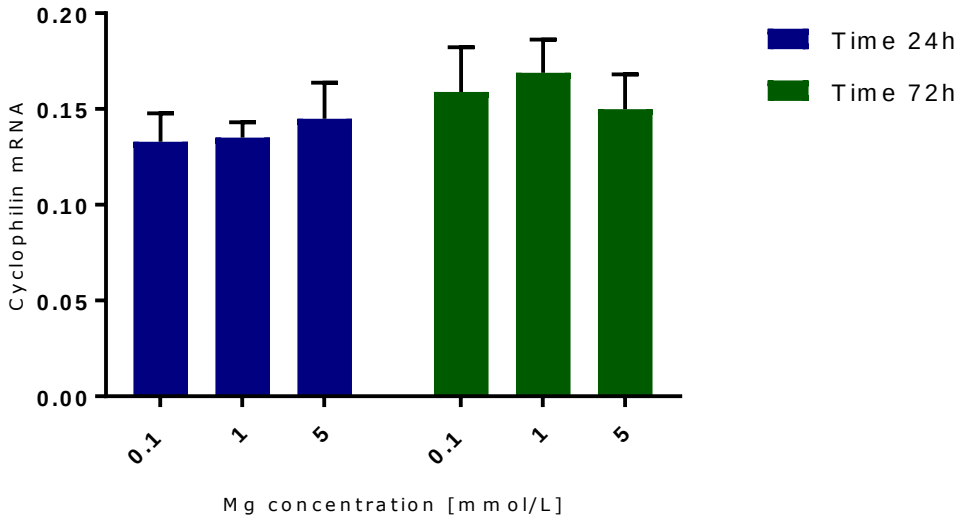
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APPENDICES

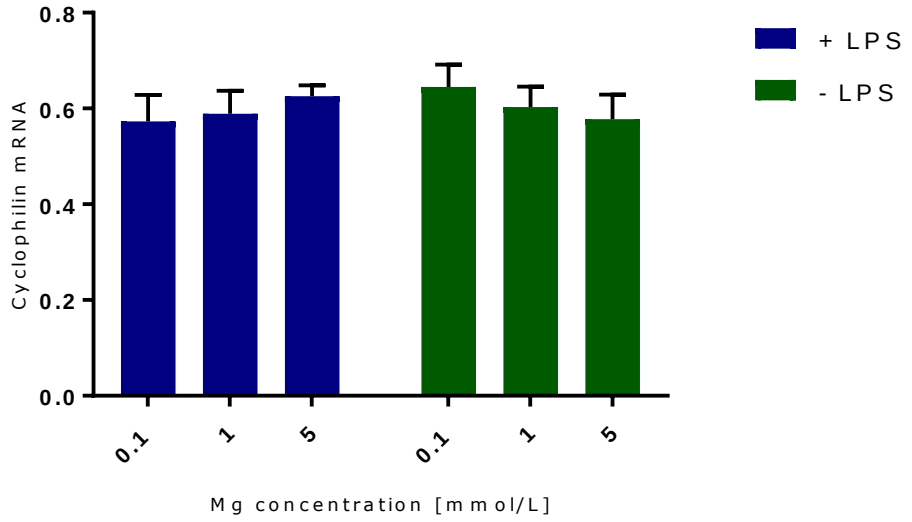
Appendix A:

Concentration and time effects of $MgSO_4$ on HUVECs expression of cyclophilin mRNA. HUVECs was cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for 24 h and 72 h. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean±SEM for n = 4. Two-way ANOVA showed no significant interaction of magnesium × time (*P*=0.646). There was no significant effect of magnesium concentration (*P*= 0.473) or time (*P*= 0.872).



Appendix B:

EffectS of different concentrations of magnesium and LPS on cyclophilin mRNA. Confluent HUVECs after 3 days of culture with $MgSO_4$ (0.1 mM, 1 mM, 5 mM) were stimulated with or without LPS (0.5 µg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data is shown as Mean±SEM for n=4. Two-way ANOVA showed no significant interaction of magnesium concentration × presence of LPS (*P*= 0.36). There was no significant effect of magnesium concentration (*P*= 879) or LPS (*P*= 0.282).



Appendix C:

Effect of different concentrations of magnesium on HUVECs expression of cyclophilin mRNA when treated or not treated with LPS and TLR antibodies. Confluent HUVECs were cultured with $MgSO_4$ (0.1 mM, 1 mM and 5 mM) for three days, and then, TLR-2 and TLR-4 blocking antibodies (10µg/ mL) were added to the HUVECs for 30 minutes before being stimulated, with or without LPS (0.5 µg/ml) for 4 hours. Total RNA was extracted and assayed by qRT-PCR. Data are shown as mean ±SEM for n=4. Two-way ANOVA showed no significant interaction of magnesium concentration × LPS/blocker (*P*= 0.358). There was no significant effect of magnesium concentration (*P*= 0.329) or blocker (*P*= 0.722).

