MECHANISMS RESPONSIBLE FOR HOMOCYSTEINE MEDIATED DAMAGE TO HUMAN ENDOTHELIAL CELLS

The role of oxidative stress in atherogenesis

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ABSTRACT

MECHANISMS RESPONSIBLE FOR HOMOCYSTEINE MEDIATED DAMAGE TO HUMAN ENDOTHELIAL CELLS

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Key words: Hyperhomocysteinemia, JNK, c-Jun, cell adhesion molecules, oxidative stress, antioxidants, nitric oxide, cytokines

Homocysteine (Hcy) has been identified as a primary risk factor for atherosclerosis as it induces endothelial cell (EC) activation/dysfunction and thus potentially initiating atherosclerotic plaque formation. There is accumulating evidence indicating a key role for oxidative stress in mediating Hcy atherogenic effects. The aim of this study was to evaluate the effects of chronic treatment with Hcy on EC activation and to explore the role of oxidative stress in these effects.

Human umbilical vein endothelial cells (HUVEC) were cultured and treated chronically with DL-Hcy for 5-9 days. An *in vitro* flow system was also used to characterize the different types of interactions between DL-Hcy-treated HUVEC and neutrophils under physiological flow conditions. EC activation was studied by characterizing the activation of the JNK pathway and the up-regulation of different cell adhesion molecules (CAM) and cytokines, using different techniques including western blot, immunohistochemical staining, enzyme-linked immunosorbent assay and polymerase chain reaction. The role of oxidative stress was investigated by measuring the production of ROS and evaluating the efficiency of antioxidants. Furthermore, the role of nitric oxide and nitric oxide synthase in modulating Hcy effects was investigated.

Chronic treatment with DL-Hcy did not kill the EC however, it inhibited cell proliferation. Furthermore, this treatment induced EC activation/dysfunction which was characterized by sustained activation of the JNK pathway, which in turn mediated up-regulation of E-selectin, ICAM-1 and to lesser extent P-selectin. Furthermore, DL-Hcy induced production of IL-8 protein. These CAM and chemokines collectively mediated different interactions between DL-Hcy-treated HUVEC and neutrophils under flow conditions including tethering, rolling, adherence and transmigration. DL-Hcy was also shown to induce significant ROS generation which mediated activation of the JNK pathway. Antioxidants restored DL-Hcy-induced interactions under flow to the basal level. DL-Hcy was shown to induce eNOS uncoupling which mediated, at least in part, the DL-Hcy-induced ROS production. Furthermore, short term treatment with NO inhibited DL-Hcy-induced HUVEC:neutrophil interactions in a cGMP-independent manner.

In summary, this research showed that DL-Hcy has several proatherogenic effects, mediated at least in part by the JNK pathway, and induces EC activation/dysfunction priming for atherosclerosis initiation. The data supports that oxidative stress mediates the majority of Hcy atherosclerotic effects. Antioxidants tested, JNK inhibitors and NO showed promising results in reversing all DL-Hcy effects and restoring EC normal status.

CONFERENCE ATTENDANCE AND PRESENTATIONS

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Submitted papers

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"I would like to dedicate this thesis to the memories of my Mum who instilled in me the drive and determination to be a better man"

Kenan

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"A little knowledge that acts is worth infinitely more than much knowledge that is idle" *Kahlil Gibran*

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"At the age of six I wanted to be a cook. At seven I wanted to be Napoleon. And my ambition has been growing steadily ever since" *Salvador Dali*

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“Friendship is always a sweet responsibility, never an opportunity” *Kahlil Gibran*

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GLOSSARY OF TERMS

Ab	Absorbance
ADMA	Asymmetric dimethylarginine
ADP	Adenosine di-phosphate
AMP	Adenosine mono-phosphate
AP-1	Activating protein–1
ATF	Activating transcription factor
ATP	Adenosine tri-phosphate
BAEC	Bovine aortic endothelial cells
BH2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
BHMT	Betaine:homocysteine methylatransferase
BSA	Bovine serum albumin
Ca^{++}	Calcium
CaM	Calmodulin
CAM	Cellular Adhesion Molecules
CBS	Cystathionine-β-synthase
CD	Cluster differentiation
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
CVD	Cardiovascular disease
Cys	Cysteine
DAPI	4’,5-Diamidino-2-pheny
DCF	2’, 7’-dichlorofluorescin
DCHF-DA	2′,7′-Dichlorofluorescin diacetate
DDAH	Dimethylargenine dimethylaminohydrolase
dH2O	Deionized water
DHF	Dihydrofolate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbeccos phosphate buffered saline
DPTA	Dipropylenetriamine NONOate
DTT	Dithiothreitol
EC	Endothelial cells
ECL	Electrochemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EF	Elongation factors

EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERT	Estrogen Replacement Treatment
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptors
GPx	Glutathione peroxidase
GSH	Glutathione
GSNO	S-Nitrosoglutathione
h	Hours
H_2O_2	Hydrogen peroxide
HAEC	Human aortic endothelial cells
HBSS	Hanks balanced salt solution
HBSS	Hanks balanced salt solution
Hcy	Homocysteine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hHcy	Hyperhomocysteinemia
HRP	Horseradish peroxidase
HS	Human serum
HSVEC	Human saphenous vein endothelial cells
HUAEC	Human umbilical arterial endothelial cells
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular adhesion molecule-1
IFN	Inteferone
IFN-γ	Interferon-gamma
IgSF	Immunoglobulin superfamily
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRE	inositol auxotrophy
JNK	c-Jun N-terminal kinases
kDa	Kilodalton
LAD	Leukocyte adhesion deficiency
LAF	Lymphocyte function-associated antigen
LDL	Low-density lipoproteins

LFA-1	Lymphocyte function-associated antigen 1 integrin
L-NAME	N (G)-nitro-L- arginine methyl ester
Lp(a)	Lipoprotein (a)
MAC-1	Macrophage differentiation antigen-1
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
min	Minute
mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
MS	Methionine synthase
mtDNA	Mitochondrial deoxyribonucleic acid
MTHF	N-5-methyltetrahydrofolate
MTHFR	5,10-methylenetetrahydrofolate reductase
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitrotetrazolium blue
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NSF	N-ethylmaleimide sensitive factor
NTP	Nucleoside Triphosphates
O2•−	Superoxide anion
ODQ	1H-[1, 2, 4]Oxadiazolo[4, 3-a]quinoxalin-1-one
OH·	Hydroxyl radical
ONOO	Peroxynitrite
PAF	Platelets-activating factor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PSGL-1	P-selectin glycoprotein ligand-1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RQ	Relative quantification
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAPK	Stress-activated protein kinases

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
siRNA	Small interfering ribonucleic acid
SMC	Smooth muscle cells
SMC	Smoothe muscle cells
SNO	S-nitrosothiols
SOD	Superoxide dismutases
THF	Tetrahydrofolate
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor alpha
tPA	Tissue plasminogen activator
TRAF	Tumor Necrosis Factor Receptor-associated Factor
u-PA	Urokinase plasminogen activator
UPR	Unfolded protein response
UV	Ultra violet
V	Volt
VCAM- 1	Vascular adhesion molecule-1
VLA-4	Very late antigen 4
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand’s Factor
WBC	White blood cells
WPB	Weibel-Palade bodies

CHAPTER 1

INTRODUCTION

1.1. THE ENDOTHELIUM AND HOMOCYSTEINE

1.1.1. Endothelial cells (EC)

Until the late1980's, the endothelium was thought to be simply a "non stick" lining of blood vessels, its only functions being to prevent blood coagulation and working as a passive barrier to diffusion of solutes and nutrients. However, it is now known that the endothelium plays more complicated roles particularly in cardiovascular physiology and pathology (reviewed by Cines *et al* (1998)) and that there are roughly 10^{14} endothelial cells in our vasculature protecting us against atherosclerosis and thrombosis (Forstermann and Munzel, 2006). In an average person, weighing 70kg, the total surface area of endothelial cells is equivalent to six tennis courts and has a total weight of about 1800g, which is a bit heavier than the liver (Born *et al.*, 1998).

Endothelial cells (EC) exist as a monolayer of specialized epithelium lining the inner surface of blood vessels, separating the vascular wall from the circulating blood and its components (Bonetti *et al.*, 2003; Lerman and Burnett, 1992). They are connected to each other by specific junctional structures at their border (Dejana *et al.*, 1995). The endothelium responds to physical and chemical stimuli, releasing substances like nitric oxide (NO), angiotensin II and reactive oxygen species (ROS) and expressing adhesion molecules like intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM- 1) and E-selectin (reviewed by Esper *et al* (2006)). Some of these substances

maintain vasomotor balance and vascular-tissue homeostasis. The importance of the endothelium in the vascular system was confirmed following the discovery that EC produce molecules that influence contractility of vascular muscle cells whose vasoactive tone determines blood flow to any organ (Highsmith, 1998). Substances produced by EC also play an important role in regulating vascular permeability, leukocyte adhesion, platelet-vessel wall interaction and vascular remodelling (Born *et al.*, 1998). EC dysfunction leads to abnormal vasodilatory responses, increased production of vasoconstrictors, impaired endothelial control of fibrinolysis and inflammation, besides altered expression of adhesion molecules (Brevetti *et al.*, 2008; Cai and Harrison, 2000). Thereafter, EC dysfunction leads to the development of atherosclerosis. At the site of injury, remnant cells can replicate rapidly and replace the lost ECs. Furthermore, circulating endothelial progenitor cells provide an additional repair mechanism for the endothelium (Asahara *et al.*, 1997).

Nitric oxide (NO) is one of the main factors required for efficient EC function, as well as vasodilatation. It is a gaseous lipophilic free radical cellular messenger whose half-life is only 10 to 20s in physiological media (Lefer, 1997). Several studies demonstrated that NO is synthesized from L-arginine and that this production is catalyzed by an enzyme called nitric oxide synthase (NOS) (Amezcua *et al.*, 1989; Furchgott, 1998; Ignarro *et al.*, 1990; Palmer and Moncada, 1989). Three different isoforms, neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS) (reviewed by Naseem (2005)). Both eNOS and nNOS produce constitutively low levels of NO in response to stimulus. While, iNOS produces large amount of NO after induction by external stimuli and can work at low levels of Ca^{2+} (Bruckdorfer, 2005). Activation of eNOS and therefore production of NO could happen

through Ca^{2+}-sensitive pathway by mediators like histamine and ATP or through Ca^{2+}-insensitive pathway by mediators like shear stress or adenosine (Fleming *et al.*, 1997).

NO activates soluble guanylyl cyclase in vascular smooth muscle, leading to accumulation of cyclic guanosine monophosphate (cGMP) and vessel relaxation (Amezcua *et al.*, 1989; Lentz, 2005; Palacios *et al.*, 1989). NO is also involved in recruiting endothelial progenitor cells which can differentiate to EC (Lowenstein, 2007). NO prevents leukocyte adhesion and subsequent migration into the subendothelial space. It can down-regulate the expression of cell surface adhesion molecules (CAM) on EC (Kosonen *et al.*, 2000; Zampolli *et al.*, 2000) and leukocytes (Kosonen *et al.*, 1999; Kubes *et al.*, 1991) resulting in anti-inflammatory effects (nitric oxide will be discussed in more details in Chapter 5).

EC responses to exogenous agents result in surface expression of several adhesion molecules, important in acute inflammation but also in disease. Therefore, their expression and regulation will be considered in more detail below.

1.1.1.1. Cellular adhesion molecules (CAMs)

CAMs are glycoproteins located on the cell surface and play an important role not only in appropriate acute inflammation but also in inflammatory as well as neoplastic diseases (Haier and Nicolson, 2000; Yamada and Geiger, 1997). They are transmembrane receptors composed of three domains: an intracellular domain which interacts with the cytoskeleton, a transmembrane domain and an extracellular domain that interacts either with other CAMs of the same kind (homophilic binding) or with different CAMs or the extracellular matrix (heterophilic binding) (Elangbam *et al.*, 1997). Interactions between

CAMs result in binding with other cells or with the extracellular matrix (ECM) resulting in cell adhesion. Four main groups of human CAMs have been recognized so far: integrins (Stewart *et al.*, 1995**),** cadherins (Elangbam *et al.*, 1997), the immunoglobulin superfamily (IgSF CAMs) (Penberthy *et al.*, 1997) and selectins (Bevilacqua, 1993).

1.1.1.1.1. Integrins

The integrins are a family of heterophilic CAMs and that are expressed by almost every cell type and they consist of two non-covalently linked molecules, alpha and beta subunits (Penberthy *et al.*, 1997). The combination of alpha and beta subunits determines ligand specificity. Integrins are also dependent on the divalent cations, Ca^{2+}, Mg^{2+} or Mn^{2+} for both structural and functional integrity (Bevilacqua and Nelson, 1993; Luscinskas and Lawler, 1994). They bind to components of the ECM extracellularly and to the cytoskeleton intracellularly, functionally connecting the cell interior to its exterior; in blood cells, integrins are also involved in cell-cell adhesion (Ruoslahti, 1991). It has been suggested that different integrins may recognize different specific domains on the same ligand and might function differently in the same cell type (Juliano and Haskill, 1993).

1.1.1.1.2. Immunoglobulin superfamily

The immunoglobulin superfamily (IgSF CAMs) consists of adhesion receptors, which share the immunoglobulin domain and include antigenic specific receptors of either T- or B-lymphocyte or both (Springer, 1994). Intercellular CAMs (ICAMs) are either homophilic or heterophilic and bind integrins or different IgSF CAMs. Human ECs express intercellular adhesion molecules ICAM-1, ICAM-2 and ICAM-3, vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressing cell adhesion molecule-1 (MadCAM-1) (Penberthy *et al.*, 1997). ICAMs help localize leukocytes to areas of tissue injury

(Elangbam *et al.*, 1997) and are constitutively expressed. However, many cytokines, such as interferon-γ (IFN-γ), interleukin-1 (IL-l), and tumour necrosis factor α (TNF-α), induce expression of ICAM on the surface of EC (Dustin *et al.*, 1986). ICAM-1 expression is believed to occur through two co-operating pathways, NF-κB-dependent and JNK-dependent pathways (Miho *et al.*, 2005). Human VCAM-1 is a cell surface protein expressed by activated EC and certain leukocytes (such as macrophages). VCAM-1 has many isoforms that differ in the number of integrin binding sites (Springer, 1994). VCAM-1 expression is induced by IL-1 beta, IL-4, TNF-α and IFN-γ with maximal activity reached by 6-12h (Bevilacqua, 1993). It regulates the adhesion of monocytes, lymphocytes, basophils, and eosinophils to EC (Elangbam *et al.*, 1997), leading to leukocyte diapedesis at the site of inflammation (Heidemann *et al.*, 2006).

1.1.1.1.3. Selectins

Selectins are a family of calcium-dependent and heterophilic cell-surface adhesion molecules. They were called selectin to highlight the presence of an amino terminal lectin domain, which is primarily responsible for their adhesive activity (Muller, 2002), as well as to emphasize the selective nature of the expression and function of these molecules (Bevilacqua *et al.*, 1989). They are expressed by leukocytes and EC and bind fucosylated carbohydrates, e.g. mucins (Bevilacqua and Nelson, 1993). Lectin domains on selectins will interact, selectively, but with low affinity, with their glycoconjugate ligands during cell-cell adhesion (McEver and Cummings, 1997). A functional synergism has been suggested between ICAMs and selectins (Steeber *et al.*, 1998). Steeber's group demonstrated in an *in vivo* study using mice that ICAM-1 expression is required to optimize selectin-mediated rolling.

Structurally, selectins are glycoprotein with an NH_2-terminal C-type lectin domain, followed by an epidermal growth factor-like domain (EGF), a series of short consensus repeats, a transmembrane domain, and a short cytoplasmic tail (Figure 1) (McEver and Cummings, 1997). Selectins are identical in their N-terminal and the lectin domain. EGF has sequence similarity while each complement regulatory-like module is about 60 amino acid length with six cysteinyl residues capable of disulfide bond formation (Bevilacqua and Nelson, 1993). The structural differences between selectins are mainly due to the number of repeated complement regulatory domains (Muller, 2002). L-selectin has two, E-selectin has six while P-selectin has nine complement protein-related domains.

The human selectin domains

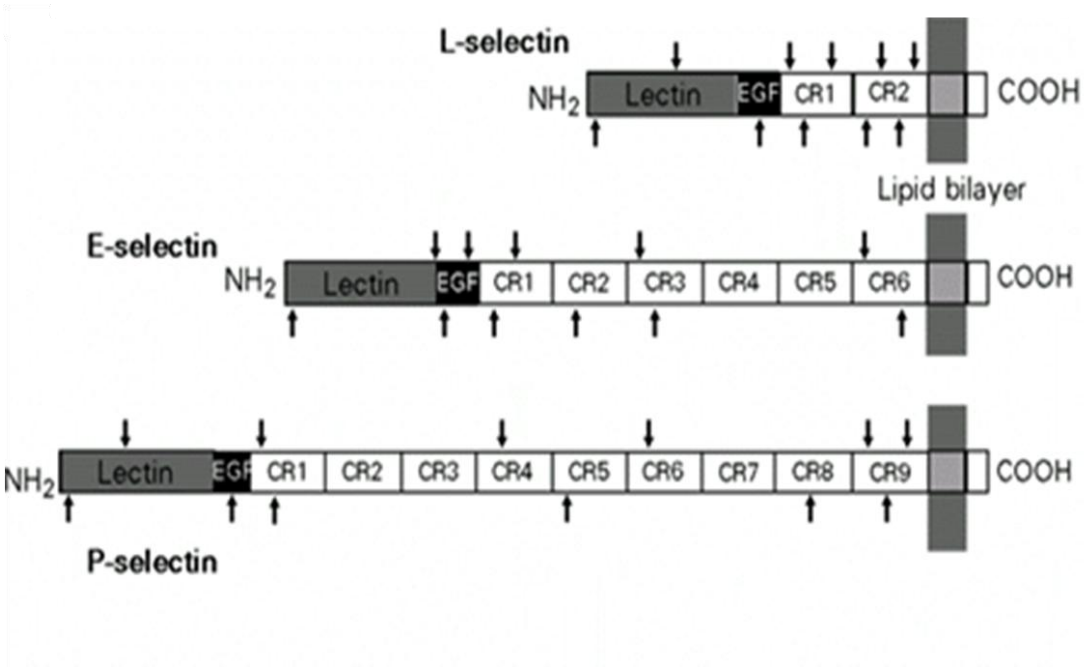


Figure 1: Human selectin domains. From left to right: amino terminal domain homologous to lectins, epidermal growth factor-like domain (EGF), complement regulatory-like modules (CR), transmembrane sequence, short cytoplasmic sequence at carboxyl terminus. Adapted from Gahmberg *et al* (1999).

A standard nomenclature has been agreed upon which designates each family member according to the cell type on which it was originally identified (Penberthy *et al.*, 1997).

1.1.1.1.3.1. E-selectin

E-selectin (CD62E), also called ELAM-1, is expressed mainly on activated EC and binds to its ligands on leukocytes (McEver and Cummings, 1997). E-selectin's expression is induced by pro-inflammatory agents including TNF-α and IL-1. It is synthesized *de novo* and expressed optimally on the EC surface 4-8h after cytokine stimulation (Bevilacqua and Nelson, 1993). The E-selectin gene is transcriptionally silent in non activated ECs. However, when these cells are stimulated, E-selectin is expressed rapidly resulting in leukocyte rolling on ECs (Whelan *et al.*, 1991). Surface expressed E-selectin will be internalized in EC but was not detected in the Golgi apparatus so it is short-lived and is degraded in lysosomes (Subramaniam *et al.*, 1993). TNF-α-induced E-selectin expression has been shown to happen through two signalling pathways. The first one involves NF-κB activation (Kaur *et al.*, 2003), while the other pathway involves JNK of the MAP kinase family which in turn leads to activation of the transcription factor ATF-2/c-Jun (Min and Pober, 1997). The E-selectin promoter contains a binding site for both ATF-2/c-Jun heterodimer and NF-κB and studies showed that both pathways are required for full activation of E-selectin gene transcription (Read *et al.*, 1997).

1.1.1.1.3.2. L-selectin

L-selectin (CD62L) is constitutively expressed on all circulating leukocytes except memory T cells (Konstantopoulos and McIntire, 1996; Tedder *et al.*, 1989) and when

expressed binds to its ligand, PSGL-1 which is expressed on EC (McEver and Cummings, 1997).

1.1.1.1.3.3. P-selectin

P-selectin has a mass of 140kDa and is considered the largest among the selectin family. It extends about 40nm from the endothelial surface. Megakaryocytes and EC constitutively synthesize P-selectin (McEver *et al.*, 1989) and store it in the alpha granules of platelets (Bevilacqua and Nelson, 1993) and in irregular cigar-shaped structures called Weibel-Palade bodies (WPB) in EC (Weibel and Palade, 1964). *De novo* synthesis of P-selectin was proven to be induced by TNF-α in mice and to peak in 3h (Weller *et al.*, 1992).

Two possible pathways for P-selectin expression have been described, immediate transient expression and late prolonged expression. The immediate one is induced by activators like thrombin and histamine and the stored P-selectin in WPB is quickly mobilized to the cell surface as rapidly as 2min (Hattori *et al.*, 1989). P-selectin expression peaks in 10min and returns back to its basal levels within a few hours even in the continuous presence of the stimulators (Blann *et al.*, 2003). Therefore, these stimulators seem to mediate acute inflammation (Khew-Goodall *et al.*, 1999). On the other hand, the expressed P-selectin in late prolonged expression is synthesized *de novo*. When P-selectin synthesis increases in response to chronic inflammation or appropriate stimulators in mice, the sorting pathways into WPB may become saturated and the extra synthesized P-selectin can be delivered directly to the cell surface (Hahne *et al.*, 1993). Once expressed on the cell surface, P-selectin binds to its ligand PSGL-1 on leukocytes (McEver and Cummings, 1997) leading to leukocyte recruitment.

1.1.1.1.3.4. P-selectin life cycle

In vivo, exocytosed P-selectin remains in the membrane for a short period (Hattori *et al.*, 1989) and is then internalised and recycled to WPB through the trans-Golgi network (Arribas and Cutler, 2000). The remnant P-selectin which fails to be internalised is shed by proteolysis (Hartwell *et al.*, 1998). It has been shown that 50% of internalised P-selectin is targeted to the lysosomes and degraded within 90min (Arribas and Cutler, 2000), while the other 50% appears in Golgi apparatus within 60-90min after P-selectin's surface expression (Subramaniam *et al.*, 1993) and is recycled to WPB. Subsequently, WPB containing recycled P-selectin start budding from Golgi apparatus and spread throughout cytoplasm within few hours (Subramaniam *et al.*, 1993). It has been shown that expression levels of P-selectin varies between different types of human EC (McEver *et al.*, 1989). HUVEC, small vessels within gastric mucosa and high EC in lymph node showed high expression of P-selectin. However, venules showed variable expression and endothelium in thyroid gland showed no expression of P-selectin (McEver *et al.*, 1989).

1.1.1.1.3.5. Selectin ligands

The main ligand for all selectins is termed PSGL-1 which stands for P-selectin glycoprotein ligand-1 (Muller, 2002) as it was initially thought to be specific for P-selectin only. However, PSGL-1 has also been shown to bind to both L-selectin (Sperandio *et al.*, 2003) and E-selectin (reviewed by Ley (2003)). Thus, the term PSGL-1 is misleading because the mucin is not a glycoprotein ligand for only P-selectin. Instead, it could be said that PSGL-1 has multifunctional properties. PSGL-1 is a 240 kDa homodimer consisting of two 120kDa polypeptide chains. It is constitutively expressed on all leukocytes and primarily found on the tips of the microvilli. It has a glycosylation pattern allowing it to

bind to endothelial selectins (McEver and Cummings, 1997). PSGL-1 structure includes the sialyl-Lewis^x component, which is expressed on neutrophils, monocytes and certain T lymphocytes (Ohyama *et al.*, 1999) and plays a key role in the recruitment of leukocytes. For optimal P-selectin and L-selectin binding, tyrosine residues on the amino terminus of the protein bearing sialyl-Lewis^x must be sulfated (Sako *et al.*, 1995; Vestweber and Blanks, 1999). A recent study in mice showed that in addition to PSGL-1, EC express another L-selectin ligand distinct from PSGL-1 that plays important role in neutrophil homeostasis and trafficking (Shigeta *et al.*, 2008).

1.1.1.1.4. Cadherins

The cadherins are a family of homophilic calcium dependent CAMs that are involved in establishing and maintaining intercellular connections (cell-cell association). Three subclasses are recognized of this family and these are E-cadherins (epithelial), P-cadherins (placental) and N-cadherins (neural) (Elangbam *et al.*, 1997). Cadherins play an important role in controlling the tight junctions between EC in the monolayer and therefore controlling the permeability (Taddei *et al.*, 2008).

1.1.1.2. Mitogen-activated protein kinase (MAP kinase)

The mitogen-activated protein kinases, MAPK, are a group of intracellular signal transduction enzymes that mediate cellular responses to external stimuli including soluble factors like growth factors, hormones and cytokines (Barr and Bogoyevitch, 2001) as well as physical factors like UV light or osmotic stress (Chang and Karin, 2001). MAP kinases, with other messenger systems work together to achieve integrated function of cells in an organism. They were identified early in evolution and are expressed in all cell types. They are involved in development processes like cell differentiation, cell proliferation, cellular

survival and death and also in controlling embryogenesis and metabolic responses in vertebrates (Barr and Bogoyevitch, 2001; Marshall, 1994; Pearson *et al.*, 2001). Many functional responses controlled by MAP kinases require information to be sent to the nucleus (Huang and Ferrell, 1996) resulting in specific phosphorylation and activation of many transcription factors, thereby modulating gene expression. The MAP kinase pathway is composed of three consecutive kinase families (termed MAPKKK, MAPKK, and MAPK). MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK (Figure 2), which phosphorylate and activate their specific target proteins such as transcription factors (Barr and Bogoyevitch, 2001; Huang and Ferrell, 1996). There are several MAPKKKs, MAPKKs and MAPKs which can be activated by diverse stimuli and significant cross talk between pathways exists. All MAP kinases have two activating phosphorylation sites in the kinase activating loop, a tyrosine and a threonine (Ferrell, 1996) so MAPK activation occurs through dual phosphorylation on Thr-X-Tyr motif (Hoefen and Berk, 2002). The activation cascade is initiated by one of a wide range of extracellular signals and each MAPK pathway has highly-specific signalling intermediates that maintain accurate signalling (Barr and Bogoyevitch, 2001). In addition, recent work has shown the importance of scaffolds in producing the appropriate response (Seeliger and Kuriyan, 2009).

Three different parallel pathways (Figure 2) have been identified with discrete functions but there is overlap in activation by certain stimuli. Extracellular signal-regulated kinase (ERK) consists of two proteins, ERK1 and ERK2 (Boulton *et al.*, 1991). They are expressed ubiquitously at different levels in different tissues (Pearson *et al.*, 2001). ERK1 and ERK2 can be activated by growth factors, cytokines, certain stresses, ligands for G

protein-coupled receptors (GPCRs), and transforming agents (Pearson *et al.*, 2001). The second MAP kinase cascade is p38, initially identified as a reactivating kinase for MAP kinase activated protein (MAPKAP) kinase-2 (Rouse *et al.*, 1994) but now known to activate a number of transcription factors (Chang and Karin, 2001). The p38 subfamily contains p38α, p38β, p38γ and p38δ (Stein *et al.*, 1997). p38 proteins are activated by cytokines, hormones, GPCRs, osmotic and heat shock, as well as other stresses (Pearson *et al.*, 2001). p38 plays an important role in the expression of some CAMs. It was shown to regulate cytokine-induced VCAM-1 expression at the postranscriptional level (Pietersma *et al.*, 1997) and also to regulate TNF-α-induced expression of MCP-1 (Goebeler *et al.*, 1999). Furthermore, activation of MAPK-activated kinase-2 (MAPKAP-2) by p38 activates heat shock protein 27, which regulates cellular morphology by uncapping actin filaments (Hoefen and Berk, 2002). Therefore, activated p38 plays a role in cytoskeleton remodeling and subsequent neutrophil transmigration following adherence to the endothelium (Wang and Doerschuk, 2001). On the other hand, p38-mediated cytoskeletal regulation is known to play an important role in response to inflammatory stimuli. Collectively, p38-mediated regulation of cytoskeletal remodeling seems to play a role in the inflammatory events.

The last MAP kinase family is termed JNK which is also known as stress-activated protein kinase (SAPK). JNK/SAPKs of 46 and 54 kDa were purified by affinity adsorption to a c-Jun fusion protein (Hibi *et al.*, 1993). Structurally, at least 10 different JNK isoforms have been identified and all of these are splice variants of JNK1, 2 and 3, known also as SAPK-γ, SAPK-α and SAPK-β, respectively (Kyriakis *et al.*, 1994). JNK1 (46 kDa) and JNK2 (54 kDa) are ubiquitously expressed, while JNK3 (54 kDa) is expressed only in the brain and testes (Barr and Bogoyevitch, 2001). Despite homology between the active sites

of JNK1, 2 and 3, each protein is the product of a different gene (Szczepankiewicz *et al.*, 2006). Like other MAP kinases, JNK/SAPKs are activated upon phosphorylation of tyrosine and threonine (Tyr^{185} Thr^{183} in JNK1), which are separated by a proline residue (Pro^{184}) (Derijard *et al.*, 1994). JNK is activated by a variety of exogenous stimuli like growth factor deprivation, pro-inflammatory cytokines such as TNF-α, UV irradiation (Zhang *et al.*, 2001a) and by all stimulants causing ER stress (Dimmeler *et al.*, 1997), for example agents that interfere with DNA and protein synthesis.

Schematic view of MAPK cascade

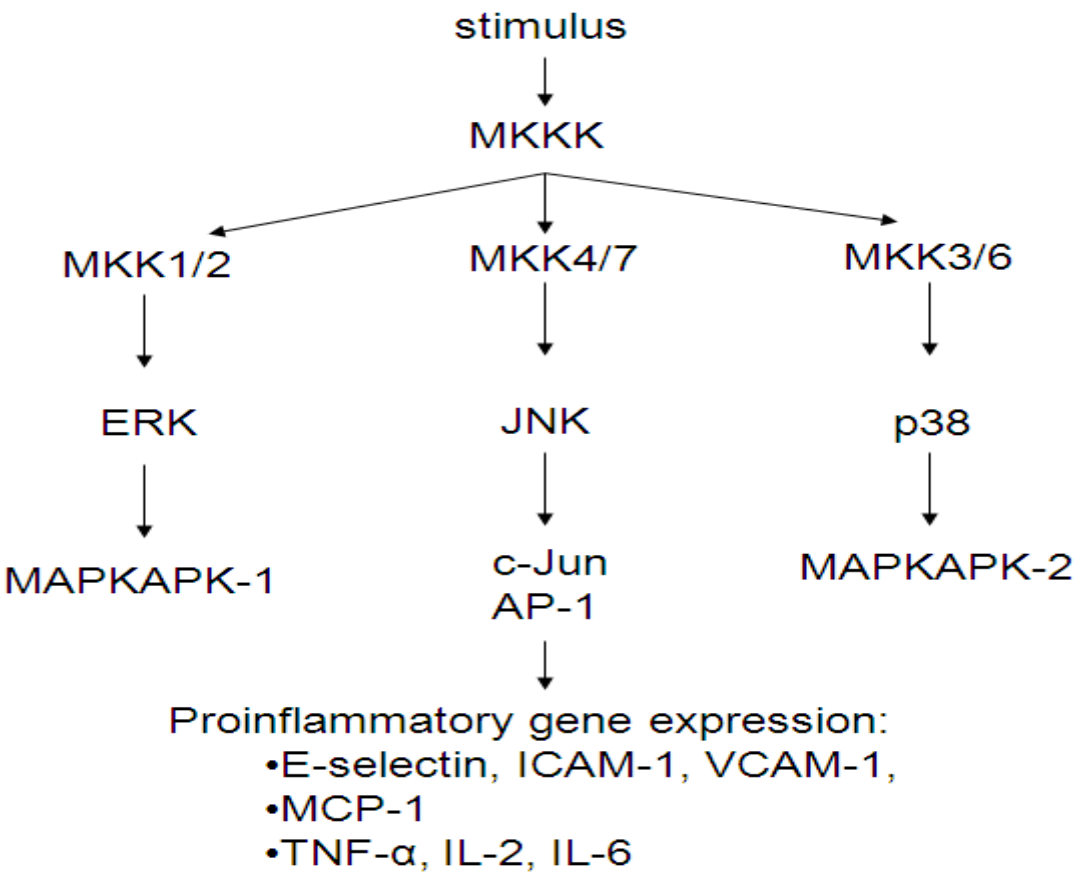


Figure 2: Schematic view of MAPK cascade. Three different parallel pathways have been identified, ERK, p38 and JNK pathway. Activation pathway of all MAPK is composed of three consecutive kinases (MAPKKK, MAPKK, and MAPK).

ASK1 is a ubiquitously expressed MAPKKK which has been shown to be a specific target for reactive oxygen species (ROS) (Shen and Liu, 2006). ASK1 activates the MAPK kinases, MKK4 and MKK7 which have been implicated in JNK/SAPK activation (Pearson *et al.*, 2001; Tournier *et al.*, 2001). *In vitro,* MKK4 preferentially phosphorylates the tyrosine residue in the activation loop motif of JNK/SAPKs, while MKK7 preferentially phosphorylates the threonine residue. Therefore, it has been suggested that these kinases cooperate to activate JNK/SAPKs (Lawler *et al.*, 1998). Different molecular targets for JNK have been identified including transcription factor AP-1 a dimer of proteins belonging to the c-Jun family: ATF-2, JunB and the insulin receptor substrate (IRS-1 and 2) (reviewed by Shen and Liu (2006)). c-Jun is specifically phosphorylated by JNK on Ser 63 and Ser 73 (Pulverer *et al.*, 1991) and this specificity was explained by specific docking requirements explaining why other mammalian MAPK members don't phosphorylate c-Jun (Barr and Bogoyevitch, 2001). c-Jun and ATF-2 are found to be constitutively bound as a heterodimer to the E-selectin promotor and their phosphorylation increases E-selectin gene transcription (Read *et al.*, 1997).

Crosstalk between different MAPK family members is a key mechanism of regulating MAPK-mediated signaling pathways. There are different pathways by which a MAPK family member can inhibit the activity of another member of MAPK family by inhibiting an upstream kinase or deactivating phosphorylation. Different studies found that some MAPK traditionally associated with stress mediated responses (JNK or p38) may inhibit the activation of another MAPK more associated with proliferation and differentiation (ERK1/2). Many studies, reviewed by Berk (2008), suggested a dynamic balance between growth factor-activated ERK1/2 and stress-activated p38/JNK pathways

may be important in determining whether a cell survives or undergoes apoptosis. Furthermore, it has been reviewed that ERK1/2 regulates the expression of MAPK phosphatase 2 (MKP2) which deactivates JNK (Hoefen and Berk, 2002). Phosphorylated p38 can also directly interact with ERK1/2 and prevent its phosphorylation by MEK1/2. Collectively, it seems that the crosstalk among JNK/p38 and ERK1/2/BMK1 may play a critical role in determining the inflammatory status of EC.

1.1.2. The role of EC in inflammation

The inflammatory process is a vital response. However, wrong timing and location might lead to potentially fatal diseases, e.g. atherosclerosis and autoimmune disease. Regulation of cell adhesion molecule expression is one mechanism to prevent an unwanted inflammatory response.

Two main forces prevent flowing blood cells from binding to the vessel, hemodynamic forces due to blood flow and adhesive forces mediated by CAMs and their ligands. Under normal conditions, neutrophils will circulate for few hours and then will be depleted by removal in the spleen (Coxon *et al.*, 1999). In addition to adhesion molecules, several soluble factors, reviewed by Konstantopoulos and McIntire (1996), have been found to be involved in leukocyte interactions with endothelium during their circulation in the blood stream; e.g. chemokines produced by endothelium like interleukin-8 (IL-8) and platelet-activating factor (PAF). Lawrence's group was the first to refer to the receptor-dependent adhesive events and defined the different stages like rolling and firm adhesion (Lawrence *et al.*, 1987). During the inflammatory response (Figure 3), adhesion molecules from circulating leukocytes interact with their ligands on the ECs lining the vessel wall, tethering them for a microsecond causing activation and then these activated leukocytes roll

on EC, adhere and finally transmigrate through the endothelial monolayer to the inflamed area.

1.1.2.1. Capture or tethering

The first contact between leukocytes and EC is termed capture or tethering. However, prior to that, margination starts when leukocytes leave the blood stream initiating leukocyte-EC interactions by close mechanical contacts. L-selectin and P-selectin expressed on activated leukocytes and EC respectively mediate tethering through binding to their counter receptor on the opposing cell (Carlos and Harlan, 1994; Muller, 2002). One report (Henderson *et al.*, 2001) suggested that leukocyte VLA-4 interaction with VCAM-1 on inflamed endothelium may have a role in tethering. Furthermore, one early study suggested that E-selectin might contribute to neutrophil tethering (Lawrence *et al.*, 1994). Selectin-ligand interaction happens quickly and detachment must also occur quickly. These interactions must be strong enough to overcome the shear forces-dissociation effects (Konstantopoulos and McIntire, 1996). Furthermore, leukocytes can attach to adherent leukocytes, and this is called secondary tethering, mainly mediated by L-selectin-PSGL-1 binding (Bargatze *et al.*, 1994) amplifying recruitment of leukocytes to the vascular wall. Furthermore, activated platelets express P-selectin which will bind to PSGL-1 and leading to more recruitment of leukocytes to the site of inflammation (McEver and Cummings, 1997).

1.1.2.2. Rolling

Leukocyte tethering leads to transient adherence to endothelium; then the next stage, rolling, occurs. Once again, the selectins mediate this stage, with the family member responsible depending on the time subsequent to inflammatory stimulus. Following the

appropriate stimulation, e.g. trauma or by histamine, P-selectin is rapidly surface-expressed on the endothelium and makes it "sticky" to leukocytes. P-selectin on the EC and L-selectin on the leukocytes, and their ligands on the opposing cells like PSGL-1, (Carlos and Harlan, 1994; McEver and Cummings, 1997) have been shown to be responsible for early stage of leukocyte rolling. Studies showed that histamine release from mast cells was indeed responsible for the "baseline rolling" seen in intravital microscopy experiments and induced by P-selectin expression (Kubes and Kanwar, 1994).

Collectively, leukocytes roll along the endothelium and during the process bonds form at the leading edge of rolling cells and are broken at the trailing edge. When P-selectin is absent, L-selectin will mediate the whole process. However, rolling velocity is 3-5 times faster in this case. Therefore, it could be said that L-selectin is much less effective then P-selectin in mediating rolling. The role of E-selectin in this process appears later *in vivo*, because it requires *de novo* synthesis and therefore is only expressed after several hours; E-selectin mediates slow rolling and initiates the next step, firm adhesion (Carlos and Harlan, 1994).

The different stages in inflammatory response

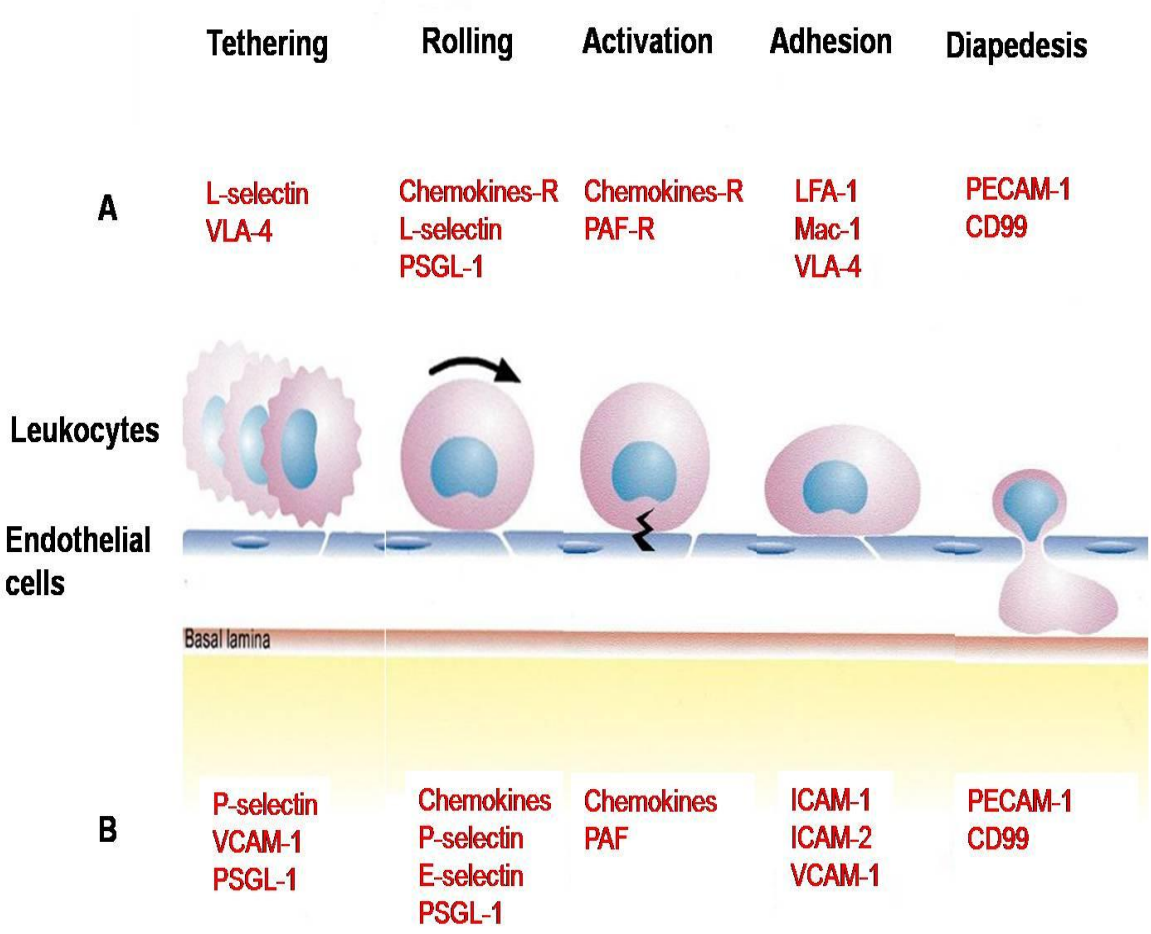


Figure 3: Sequential steps in leukocytes emigration through ECs in inflammatory response. The ligand in (A) are from leukocytes and those in (B) are from EC. Adapted from Muller (2002).

1.1.2.3. Slow rolling

This stage has been termed slow rolling to distinguish it from the much faster rolling that P-selectin is responsible for and requires E-selectin expression on EC interacting with CD18 integrins on the rolling leukocytes as studies on mice showed that exposure to TNF-α resulted in decreasing leukocyte rolling velocity to an average between 5-10μm/s (Jung and Ley, 1999; Kunkel *et al.*, 2000). Slow rolling has been shown to happen *in vitro* on E-selectin substrate and P-selectin substrate using artificial lipid bilayers containing the same amounts of the two selectins (Lawrence and Springer, 1991) meaning the slow rolling is not based on the unique property of E-selectin. However, *in vivo* P-selectin is expressed at a much lower level compared to E-selectin (Blann *et al.*, 2003) which is not enough to mediate rolling *in vivo*. Furthermore, rolling has been shown in *in vivo* studies in genetically modified mice to require the expression of E-selectin on EC (Jung and Ley, 1999). Collectively, E-selectin plays the key role in mediating slow rolling. Leukocytes need to spend a certain amount of time in a position favourable for bond formation. However, Jung *et al* have found that slow rolling is not strictly required because high concentrations of chemoattractants can arrest fast-rolling leukocytes (Jung *et al.*, 1998). Furthermore, it has been shown (Kubes *et al.*, 1995) that rolling velocity must be decreased by more than 90% to significantly reduce firm adhesion. It appears that E-selectin and CD18 integrin work synergistically to control the time a leukocyte needs to roll through an inflamed area and to convert rolling to firm adhesion (Jung *et al.*, 1998) and a correlation has been established between rolling transit time, which defines to the duration when leukocytes are rolling closely to ECs, and the amount of firm adhesion (Jung *et al.*, 1998; Kunkel *et al.*, 2000).

1.1.2.4. Firm adhesion

Rolling leukocytes do not stop abruptly, but they show a decrease in their rolling velocity allowing bonds (Blann *et al.*, 2003) to be formed between them and EC, initiating the next step, firm adhesion. The leukocytes, while they are rolling along the vessel, integrate chemoattractant signals until they reach a critical level of activation and become firmly adherent (Jung *et al.*, 1998). This step is mainly controlled by the leukocyte's integrins which bind to counter receptors which are members of the immunoglobulin superfamily on the EC (Muller, 2002; Sligh *et al.*, 1993). CD18 integrin participates in the leukocyte arrest. Neutrophils also express small amount of VLA-4 [α4β1 integrin] which can bind endothelial VCAM-1 and mediates firm adhesion (Luscinskas *et al.*, 1995). Furthermore, lymphocyte function-associated antigen 1 integrin (LFA-1) plays a key role in mediating leukocyte adherence through binding to ICAM-1 on EC. Other integrins like macrophage differentiation antigen-1 (MAC-1) are expressed on neutrophils and can bind ICAM-1 and ICAM-2 on the endothelium, however MAC-1 has been suggested to play a role in activating neutrophils and phagocytosis rather than in their adherence and LFA-1 was suggested to play more important role in leukocyte arrest (Dunne *et al.*, 2002). Despite this, the same study suggested that both LFA-1 and MAC-1 are required for leukocyte rolling on inflamed venules.

1.1.2.5. Transmigration

All of the preceding steps in leukocyte recruitment are reversible. However, diapedesis is the "point of no return". *In vitro* experiments showed that chemokines like IL-8 induced dose-dependent increases in neutrophil transmigration (Furie *et al.*, 1991). Therefore, when appropriate exogenous chemoattractant is present, like IL-8, leukocytes

squeeze between the opposing activated ECs and migrate across endothelium and undergo a change in their cytoskeleton and extend themselves across endothelial border (Muller, 2002). It is believed that this step is stimulated by chemoattractants from the inflammatory environment including factors released from necrotic host cells and chemokines made by host cells, including EC, in response to inflammatory stimuli (Muller, 2002).

While these steps have been represented in the previous paragraphs as sequential, it would be true to say that there is significant overlap at each stage and knock out studies have shown that some of the adhesion molecules can substitute for each other under certain conditions.

1.1.3. Signal transduction in endothelial cell activation

The MAPK family plays an important role in mediating the signal transduction from the cell surface to transcription factors in nucleus, resulting in alterations in gene expression (Chang and Karin, 2001). For example, cellular exposure to cytokines or environmental stress causes JNK activation and that presents a possible role of JNK in mediating the inflammatory response. Experiments showed that cytokine-stimulated cultured ECs express E-selectin. TNF-α-mediated transcription of E-selectin peaks in 2h and returns into base-line after 12h of induction with the expression of the protein peaking after 4-6h (Read *et al.*, 1997; Wadgaonkar *et al.*, 2004). Different studies in co-cultured EC/SMC (Chiu *et al.*, 2007) or in animal models (reviewed by Ip and Davis (1998)), showed that cytokine-induced E-selectin expression, is mediated by a variety of intracellular signaling intermediates including JNK, p38, and NF-κB. The role of JNK in E-selectin expression was confirmed by the reduction in its expression in the presence of the selective JNK inhibitor SP600125 in co-cultures of EC/VSMC treated with the cytokine IL-

1. TNF-α interacts with its receptor TNFR1 and results in different TNF-α-induced signals. Consequently there will be activation of JNK/p38 and NF-κB leading to activation of ATF-2 and AP-1 (Gupta *et al.*, 1996; Sluss *et al.*, 1994). In turn, this leads to activation of the E-selectin promoter and thus full gene transcription. Therefore, induction of endothelial E-selectin gene expression by inflammatory mediators is mediated by signalling pathways involving MAP kinase family members and NF-κB and this E-selectin expression confirms the role of the MAPK family in mediating inflammatory response. The roles of JNK in mediating expression of adhesion molecules in response to diverse stimuli are still unclear and different studies are underway to clarify such functions of MAP kinases.

1.1.4. EC and the development of atherosclerosis

Endothelial dysfunction involves different abnormalities in the homeostatic level of vascular endothelium including disturbed vascular tone, haemostasis, and inappropriate inflammation (Cai and Harrison, 2000). These abnormalities lead to impairment of endothelium-dependent relaxation of blood vessels, ultimately resulting in cardiovascular disorders (Schachinger *et al.*, 2000). Deanfield *et al* (2007) proposed that under certain circumstances, eNOS can induce the production of ROS in a process known as eNOS uncoupling. The low levels of either the cofactor BH_4 or the substrate L-arginine induces eNOS uncoupling and the formation of superoxide or hydrogen peroxide respectively (Forstermann and Munzel, 2006). Furthermore, under inflammatory conditions, as in atherosclerosis, activated leukocytes are attracted to the endothelial site of injury and generate significant amounts of ROS which worsen the injury. Under normal conditions, ROS play a role in diverse signalling pathways and they are involved in regulating vascular tone, cell growth and apoptosis (Papatheodorou and Weiss, 2007). In addition to generating

ROS, EC have scavenging mechanisms like antioxidant enzymes that prevent build-up of ROS and damage. However, in different cardiovascular diseases, ROS overcome antioxidant defences resulting in what is known as oxidative stress (Davidson and Duchen, 2007). The early events of atherosclerosis include sublethal changes in EC where the endothelium shows increased permeability to plasma proteins and lipoproteins, like LDL, albumin and fibrinogen. LDL binds to extracellular matrix proteins of the subendothelial space and accumulates in the artery wall, where it can be oxidised. Subsequently, leukocytes are recruited to the lesion area and interact with EC, tether, roll, adhere and migrate to the subendothelial space. Activated EC produce growth factors like macrophage colony-stimulating factor which induces activation and differentiation of transmigrated monocytes to resident macrophages (Lusis, 2000). These macrophages in turn internalize oxidized LDL (Lusis, 2000) forming lipid-laden foam cells, the hallmark of the fatty streak (Frei, 1999). EC and leukocytes release different chemokines that cause proliferation and migration of smooth muscle cells (SMC) from the media to the intima. SMC help in accumulation of collagen and proteoglycan which are essential factors for the plaque stability (Braunersreuther *et al.*, 2007). Two phenotypes of plaque have been identified, those with thick fibrous cap and small lipid core and termed stable plaques and they do not pose much danger of inducing atherosclerosis complications. Unstable plaques are characterized by large lipid core and thin fibrous cap and are prone to rupture inducing thrombosis leading to acute vascular events (Fuster *et al.*, 1999; Tedgui and Mallat, 2006).

Collectively, chronic exposure to cardiovascular risk factors activates different molecular pathways in EC leading to the expression of different cytokines, chemokines and CAM (Deanfield *et al.*, 2007). Consequently, these ECs will become dysfunctional and lose

their vasoprotective functions. Furthermore, a number of risk factors like TNF-α can induce EC apoptosis which leads to loss of the protective function of EC. Severely damaged ECs play a primary role in the initiation of atherosclerosis by a variety of mechanisms that are still under investigation.

1.1.5. Homocysteine

1.1.5.1. Homocysteine chemistry

Homocysteine (Hcy) is a sulphur-containing amino acid (Ling and Hajjar, 2000); it is not present in naturally occurring proteins and is not a dietary constituent (Bolander-Gouaille, 2002). Therefore, all Hcy found in organisms is primarily derived from demethylation of dietary methionine (D'Angelo and Selhub, 1997; Scalera *et al.*, 2006). Hcy can form a disulphide bond, through its thiol group, with another homocysteine molecule forming homocystine, with free cysteine (Cys), or with the thiol group of plasma proteins like albumin (Medina *et al.*, 2001). Normally, 1% of Hcy exists in the plasma in the free reduced form and the rest in oxidized form. About 70 % of Hcy is bound to albumin and the remainder forms disulphides (Bolander-Gouaille, 2002). Hcy and Cys have the same chemical structure as methionine with one and two methyl groups less respectively (Figure 4).

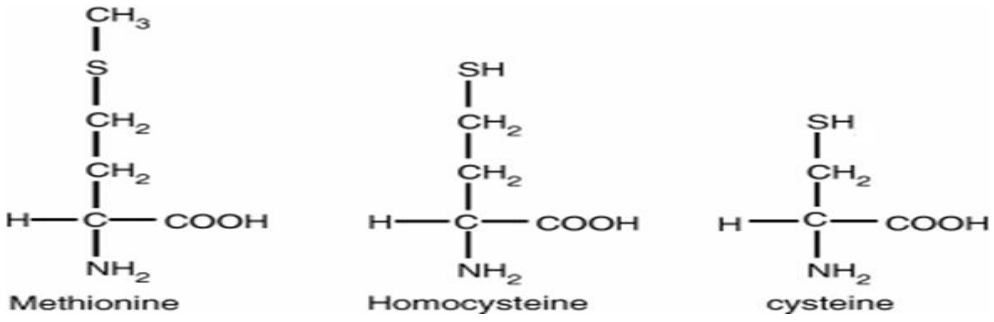


Figure 4: The chemical structure of methionine, homocysteine and cysteine.

1.1.5.2. Hcy transportation to the cell and its metabolism

Amino acids use different systems for transport into the cell which can be divided into two major groups, Na^+-dependent and Na^+-independent amino acid transporters. In addition to sodium ions, other different factors may regulate the transporter systems like pH, size of the amino acid and the ionic charge of the amino acid side chain (reviewed by Mann et al (2003)). Hcy is a neutral amino acid and its transport is pH insensitive (Ewadh *et al.*, 1990). Its transport into the cells is essential for Hcy signalling and to induce its cellular effects. Ewadh *et al* showed, using [^{35}S] Hcy, that Hcy cellular uptake in HUVEC happens through at least two different systems, the alanine-serine-cysteine (ASC) (Na^+-dependent) system and the large branched-chain neutral amino acid system (system L) (Na^+-independent) both of which are also responsible for transporting Cys (Ewadh *et al.*, 1990). Furthermore, a recent study found that two additional sodium-dependent transporter systems X_{AG} and A are involved in Hcy binding and uptake by HAEC (Budy *et al.*, 2006). However, transport system ASC has been suggested to dominate Hcy transport in EC (Budy *et al.*, 2006; Ewadh *et al.*, 1990). Ewadh *et al* (1990) found that including Cys in the media inhibited Hcy uptake and suggested that Hcy transport happens through one of the systems used to transport Cys. They suggested that the inhibitory effects of cystine and homocystine on the uptake of the reduced form Hcy could be due to the formation of mixed disulphides.

Methionine is converted to Hcy through two mediator compounds (Figure 5), S-adenosylmethionine (SAM) which is the methyl donor for most cellular methyltransferase reactions. SAM in turn is demethylated to S-adenosylhomocysteine (SAH). Resulting Hcy is remethylated through the methionine cycle which is mediated by methionine synthase,

vitamin B12, methyltetrahydrofolate reductase (MTHFR) and folate (Lawrence de Koning *et al.*, 2003). Hcy might also be metabolized through an irreversible reaction to cysteine through the transsulphuration pathway as animal cells cannot synthesize Hcy from Cys (Finkelstein, 1998). The transsulphuration pathway is mainly mediated by cystathionine-β-synthase (CBS) with its cofactor vitamin B6 and it is the only reaction that removes Hcy from the methionine cycle (Finkelstein, 1998). These two overlapping pathways (Figure 5), transsulphuration and remethylation, are responsible for elimination of Hcy (D'Angelo and Selhub, 1997; Ling and Hajjar, 2000). Specific cellular Hcy export mechanisms are responsible for depositing excess Hcy into the blood and maintaining the intracellular level of Hcy at a low intracellular concentrations limiting intracellular toxicity (D'Angelo and Selhub, 1997). The kidney plays an important role in Hcy clearance mainly through the transsulphuration pathway (Bostom and Lathrop, 1997) and that explains why people with renal failure have a high incidence of vascular complications as they will have elevated Hcy levels (Ji and Kaplowitz, 2004). Hcy can also be deaminated in the liver where the amino group is removed and converted to ammonia which in turn will be converted to urine or uric acid (Medina *et al.*, 2001). Furthermore, in the liver and the kidney, some Hcy is methylated to methionine by the enzyme betaine:homocysteine methyltransferase (Selhub, 1999). SAM was suggested to have a regulatory role in Hcy metabolism as Mato *et al* (2002) illustrated that SAM inhibits MTHFR and betaine:homocysteine methylatransferase (BHMT) and activates CBS. Low SAM favours remethylation and directs Hcy for methionine synthesis, while high SAM favours transsulphuration.

Homocysteine metabolism

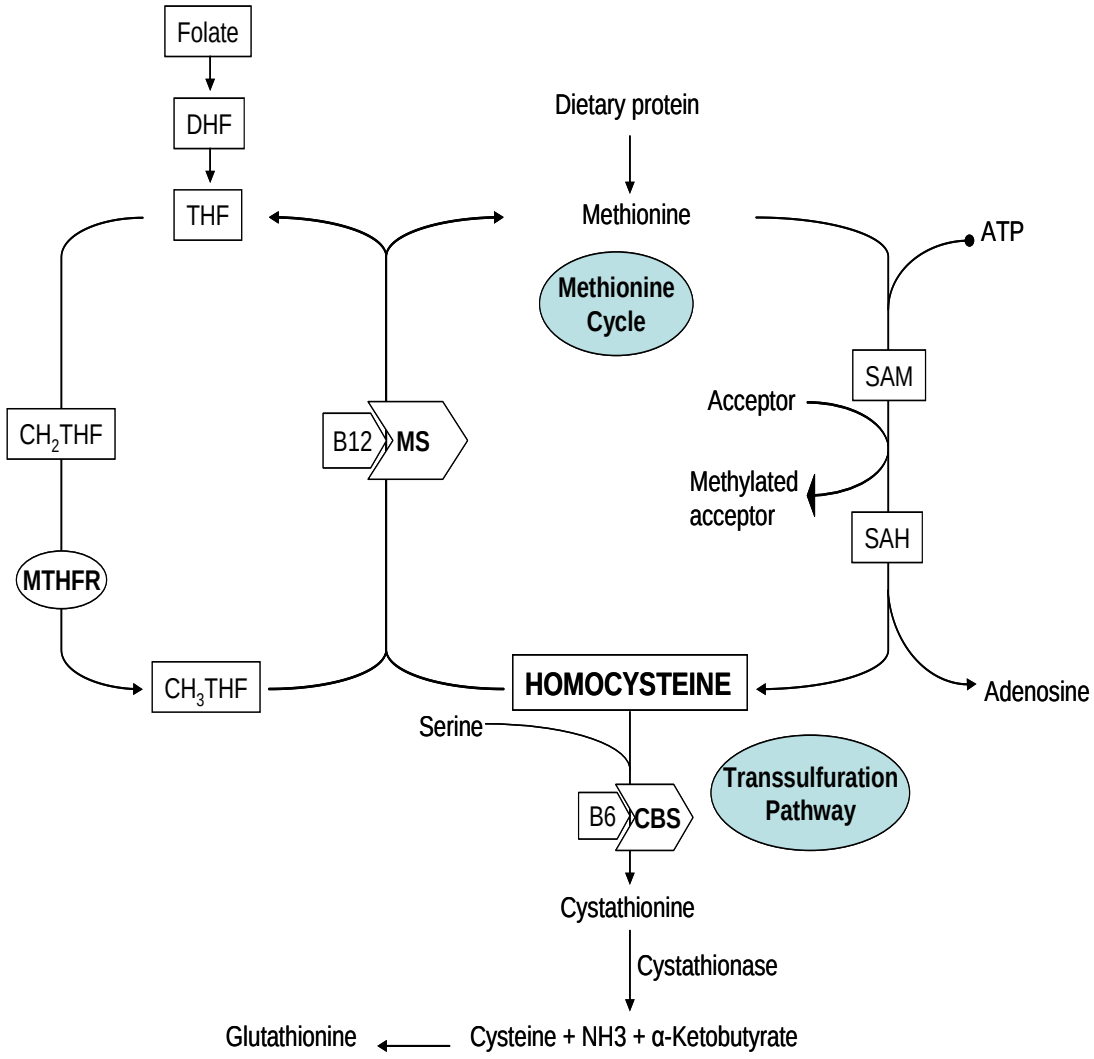


Figure 5: Homocysteine metabolism: DHF: dihydrofolate, THF: tetrahydrofolate, CH_2THF: methylene tetrahydrofolate, CH_3THF: methyl tetrahydrofolate, MTHFR: methyltetrahydrofolate reductase, MS: Methionine synthase, CBS: cystathionine β synthase, SAM: S-adenosylemethionine, SAH: S-adenosylehomocysteine.

1.1.5.3. Hyperhomocysteinemia

Abnormal metabolism of Hcy could be due to defects in either demethylation or transsulphuration pathways and results in a disease called hyperhomocysteinemia (hHcy) (den Heijer *et al.*, 1996). hHcy could also be caused by nutritional defects, renal dysfunction, alcoholism, hyperthyroidism, or certain medications (reviewed by Lentz and Haynes (2004)). Three ranges of hHcy have been defined: moderate (16-30 μM) which occurs in 5-7% of the population (Lawrence de Koning *et al.*, 2003), intermediate (31-100 μM) and severe (> 100μM) (Ji and Kaplowitz, 2004; Lentz and Haynes, 2004). hHcy has been reported to be accompanied by other abnormalities like cardiovascular diseases (Lawrence de Koning *et al.*, 2003), osteoporosis (van Meurs *et al.*, 2004), dislocation of ocular lens, neural tube defects and mental retardation (D'Angelo and Selhub, 1997; Selhub, 1999).

The remethylation pathway required for Hcy metabolism could be impaired due to inadequate status of either folate or vitamin B12 or due to a defect in the gene encoding for N-5-methyltetrahydrofolate (MTHF) (Kang *et al.*, 1988; Zhang *et al.*, 2001a). Such genetic defect leads to a significant increase of Hcy in plasma. MTHFR deficiency might result from a point mutation, C to T substitution at nucleotide 677 (C677→T), in the open reading frame of MTHFR gene. This mutation causes substitution of valine for alanine in the functional enzyme, resulting in a thermolabile variant of the enzyme with decreased total activity (reviewed by Ji and Kaplowitz (2004)). The frequency of this mutation varies among ethnic groups with 13% of homozygous and 50% of heterozygous among Caucasian and Asian populations. On the other hand, impairment in the transsulphuration pathway could result from hetereozygous defects in CBS gene or insufficient levels of vitamin B6

(Kokame *et al.*, 1996; Selhub and Miller, 1992). Several mutations in human CBS have been defined (D'Angelo and Selhub, 1997), the most frequent are T833→C and G919→A, located in the exon 8 and inherited as an autosomal recessive disorder, and 1224-2AC, which cause exon 12 to be deleted. Homozygous CBS deficiency is a rare syndrome which results in plasma Hcy concentration of up to 400μM and pleiotropic clinical manifestations, including mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities and hepatic steatosis (reviewed by Ji and Kaplowitz (2004)). However, heterozygous CBS deficiency is relatively common with a prevalence of 1% of the population (Boers *et al.*, 1985; Lawrence de Koning *et al.*, 2003).

Hcy concentration in the plasma may differ depending on which of these two pathways is defective. Several studies reported that the worst complications were observed in patients with defective MTHFR (reviewed by D'Angelo and Selhub (1997)). Furthermore, although other studies reported that MTHFR-deficient mice have a similar basic phenotype to those with CBS-deficiency, they showed higher susceptibility to the development of atherosclerosis (Chen *et al.*, 2001b; Lawrence de Koning *et al.*, 2003). Methylation is the main pathway for elimination of Hcy in human vascular tissues while transsulphuration pathway enzymes were shown not to be expressed in these tissues (Chen *et al.*, 1999). That might explain the above described results by Chen *et al* (2001b) and Lawrence de Koning *et al* (2003). Toxicity of hHcy is a result of different Hcy metabolites like Hcy thiolactone (HcyT), S-nitroso-Hcy (Jakubowski, 2004) in addition to protein homocysteinylation which all may lead to protein damage either directly or indirectly (Jakubowski *et al.*, 2000).

In addition to methylation and transsulphuration metabolic pathways of Hcy, intracellular Hcy could be converted by methionyl tRNA synthase to Hcy-AMP complex which is in turn catabolised to Hcy thiolactone, which could be considered as an alternative pathway for the conversion of Hcy into methionine (Medina *et al.*, 2001) and indicates a sign of poor methylation of Hcy-tRNA to methionine-tRNA. Hcy thiolactone, due to its reactive properties, can lead to homocysteinylation of lysine residues and free amino groups of cellular protein decreasing their biological activity and causing them to degrade. Hcy thiolactone is also secreted into the circulation and causes modification of some plasma proteins which might contribute to liver and cardiovascular disorders (reviewed by Ji and Kaplowitz (2004)).

1.1.5.4. Pathogenesis of Hcy effects

1.1.5.4.1. Cardiovascular diseases and Hcy involvement

Cardiovascular disease (CVD) refers to any disorder which affects the cardiovascular system and mainly to the atherosclerosis-related diseases such as myocardial infarction and stroke. Atherosclerosis is initiated at the site of vascular injury on the level of EC (as explained in section 1.1.4), and progresses in a silent manner until the formation of stratified lesions in the arterial walls, which contain cholesterol, immune infiltrate and connective tissue elements (Braunersreuther *et al.*, 2007), leading to acute events like heart attack (Lawrence de Koning *et al.*, 2003). Different risk factors are involved in the progress of atherosclerosis such as gender (Jousilahti *et al.*, 1999), lipid levels, diabetes, blood pressure, diet, smoking, alcohol (reviewed by Vinereanu (2006)). Some biomarkers also have been considered as risk factors including fibrinogen, homocysteine, asymmetric dimethylarginine (ADMA). All of these factors are known to cause EC damage and

dysfunction (Celermajer, 1997) and different pharmacologic approaches and diet changes have been suggested in order to reverse the damage of ECs (Mensah *et al.*, 2007).

hHcy was reported few decades ago to be involved in the development of CVD. In 1964, Gerritsen and Waisman first reported a fatal pulmonary thromboembolism in a one year old child with hHcy (Gerritsen and Waisman, 1964). The second observation was made by McCully in 1969 when he reported the death of an infant with homocystinuria who had widespread severe arteriosclerosis (McCully, 1969). From these data, the Hcy theory of arteriosclerosis was formulated (McCully and Wilson, 1975). In 1976, Wilcken and Wilcken showed that, compared with normal subjects, those with coronary artery disease exhibited higher plasma Hcy levels following a challenge with oral methionine (up to 30µM of Hcy-Cys mixed disulfide) (Wilcken and Wilcken, 1976). Since that, a number of groups have shown that up to 40% of patients diagnosed with coronary, cardiovascular, or peripheral atherosclerosis have hHcy (D'Angelo and Selhub, 1997; Lawrence de Koning *et al.*, 2003; Selhub *et al.*, 1995) and it has been shown that mild increase in Hcy (3µM above normal range) may be associated with higher risk of atherosclerosis (Nygard *et al.*, 1998; Tsai *et al.*, 1994), stroke (Lentz, 2005) and deep-vein thrombosis (den Heijer *et al.*, 1996). In addition, a decrease of 25% in Hcy has been associated with an estimated 10% lower risk for CVD or 20% less for stroke (Herrmann *et al.*, 2003).

Different circulating concentrations have been used to define hHcy, 15.8µM (Stampfer *et al.*, 1992), 13.9µM (Joosten *et al.*, 1993), 11.4µM for male and 10.4 µM for female (Selhub *et al.*, 1995). Currently, the American Heart Association science advisory statement suggested that 10µM or less is a reasonable target for patients at high cardiovascular risk. However, a European expert panel has proposed a slightly higher target

level (13–15 μM) (reviewed by (Lentz and Haynes, 2004)). Different factors have been found to affect these suggested reference ranges for example, increasing age (Bates *et al.*, 1997; Selhub *et al.*, 1999), low folate intake (Nygard *et al.*, 1998), smoking and caffeine consumption.

The contribution of Hcy to the onset of cardiovascular diseases has been explained by several pathogenesis mechanisms. The most widely accepted is the oxidative stress theory which will be discussed in detail in Chapter 4. hHcy has been found to induce several genetic alterations as Ling and Hajjar (2000) showed that when EC were treated 6 – 42h with Hcy, these cells exhibited significantly increased steady state mRNA levels for the elongation factors (EF) EF-1α, β and δ, and a reduction in protein levels. EF1 is a transcription factor that appears to play a general role in cell growth, proliferation and differentiation (Proud, 1994). Outinen (1999) found that Hcy also alters the expression of number of genes, which are known to mediate cell growth and differentiation, for example GADD45, GAD153, ATF4, and YY1, suggesting that Hcy causes a dose-dependent decrease in DNA synthesis in HUVEC and so causes a dose-dependent decrease in EC growth. Furthermore, Dardik *et al* (2002) found that under physiologic flow conditions, Hcy influences gene/proteins mediating cytoskeleton organization, for example it up-regulates profilin-I and down-regulates endothelial cell-cell adhesion molecule alpha-catenin.

Hcy was also suggested to interfere with the fibrin elimination process by impairing EC ability to generate plasmin *in vitro* (Chacko *et al.*, 1998; Hajjar *et al.*, 1998). Furthermore, different studies reported increased affinity of Lp(a) for fibrin and strengthened Lp(a) effect in the presence of homocysteine (Harpel *et al.*, 1992; Sotiriou *et*

al., 2006). Collectively, Lp(a) induces recruitment of inflammatory cells to activated EC in addition to accumulation of fibrin and both effects help in the build up of the atherosclerotic plaque.

Hcy has been implicated in ER stress through increasing the expression of several endoplasmic reticulum (ER) stress response genes like GRP78, GRP94, Herp and RTP (Ji and Kaplowitz, 2004; Kokame *et al.*, 1996). Excess Hcy might disturb disulphide bond formation and protein folding leading to ER stress (Kokame *et al.*, 1996; Outinen *et al.*, 1998; Outinen *et al.*, 1999) and thus inducing the unfolded protein response (UPR) to enhance cell survival (reviewed by Lawrence de Koning *et al* (2003)). However, failed UPR induction and prolonged ER stress can induce alteration in several cellular functions like dysregulation of lipid metabolism, apoptotic cell death, and inflammation which in turn might lead to vascular injury (reviewed by Austin *et al* (2004)). Furthermore, it has been suggested that Hcy-induced UPR leads to overproduction of ER lipid components and the accumulation of these products in affected cells (Lawrence de Koning *et al.*, 2003). This suggestion was linked to the fact that lipid rich atherosclerotic lesions are developed in patients with hHcy despite their normal serum lipid levels.

All of these possible mechanisms in addition to other contributing mechanisms like smooth muscle cell proliferation, increased collagen production and platelet aggregation might account for Hcy effects on increasing susceptibility to CVD. It could be said that one mechanism cannot account for the whole effects of Hcy and these mechanisms are working in parallel to induce the EC damage and the initiation of atherosclerosis.

1.1.5.4.2. Hcy lowering therapy in clinical trials

Several clinical trials reviewed by Loscalzo (2006), such as vitamin intervention for stroke prevention (VISP) (Toole *et al.*, 2004), the Norwegian vitamin trial (NORVIT) (Bonaa *et al.*, 2006) and Heart outcomes prevention evaluation (HOPE) (Lonn *et al.*, 2006) investigated the use of vitamin supplements, such as B6, B12 and folate, to enhance Hcy metabolism and therefore, to reduce its level to the normal range. However, these trials found that vitamin based lowering of plasma homocysteine was of no benefit in reducing cardiovascular risk. These findings contradict the findings of most published *in vitro* and *in vivo* studies about the positive correlation between Hcy and CVD.

Different possible explanations could account for this controversy. Firstly, folic acid, which was used in these trials, can induce cell proliferation by inducing thymidine synthesis (Loscalzo, 2006). Therefore, folate might have the same effects in the atherosclerotic plaque, thus worsening the injury. In addition, the use of folate and vitamin B12 will enhance the remethylation of Hcy to methionine and thereafter, this methionine will be converted into SAM (Figure 5). SAM is an essential methyl donor for all methylation reactions in the cell (Lu, 2000). The increase in SAM level leads to increase in the methylation potential, which in turn increases cell proliferation, modifies gene expression and therefore, affects endothelial function. Collectively, all of these effects might contribute to the atherosclerotic plaque formation. Furthermore, methylation of l-arginine will generate asymmetric dimethylarginine ADMA which in turn, inhibits NOS and therefore decreases the bioavailable NO, which is associated with an increased risk of vascular disease (Boger, 2003; Boger *et al.*, 2001). Another possible explanation for the low efficiency of vitamin lowering therapy in reversing Hcy effects could be due to EC,

after being chronically exposed to high level of Hcy in hyperhomocysteinemic patients, having developed some kind of sensitivity toward Hcy. Therefore, Hcy, even at low levels, due to vitamin treatment, may be still effective in keeping the EC activated. Alternatively, it could be suggested that chronic exposure to Hcy for many years might have induced irreversible damage to the EC, and therefore removing Hcy would not be able to restore EC functions. The outcomes of these clinical trials might highlight a fact that there is a need to develop other therapeutic approaches to treat the consequences of high Hcy levels, such as inhibition of the pathways involved in mediating Hcy effects or restoring normal function and/or structure of enzymes which have been altered by Hcy.

1.1.5.4.3. Homocysteine and endothelial cell dysfunction

The first *in vivo* evidence that Hcy can alter EC function and predispose to complications of vascular diseases was published in the *JCI* in 1996 (Lentz *et al.*, 1996). They reported in monkeys with diet-induced hHcy that a two-fold increase in Hcy altered vasomotor regulatory function, EC anticoagulant function in addition to impairing endothelium-dependent vasodilation. A positive correlation between plasma homocysteine levels and age has since been demonstrated (Nygard *et al.*, 1998). Subsequently, Xu *et al* showed that Hcy can accelerate aging, which is often defined as replicative senescence, in cultured human HUVEC through a redox-dependent pathway (Xu *et al* 2000) and it has also been shown to induce necrosis in HUVEC (Dong *et al.*, 2005). Further studies using different models like genetic- or diet-induced hHcy in animal models and Hcy-treated EC in culture strongly support a relationship between hHcy and EC dysfunction (reviewed by Austin *et al* (2004)).

Because it has been proven that in the human cardiovascular system Hcy metabolism is limited to the remethylation pathway catalyzed by MS (Chen *et al.*, 1999), atherosclerotic initiation could be due to either failure of these patients' EC to detoxify endogenous Hcy due to lack of MS efficiency or from the direct toxicity of high concentrations of extracellular Hcy (Finkelstein, 1998).

Several studies, reviewed by Jakubowski (2004), showed that Hcy has inhibitory effects on the growth rate of yeast and *E. coli* cells. Furthermore, Zhang *et al* found that Hcy does not support growth and induces apoptotic death in human EC (Zhang *et al.*, 2001a). Therefore, Jakubowski suggested that Hcy interferes with biological processes in different types of live cells with a common consequence of inhibiting their proliferation (Jakubowski, 2004). Even though some studies reported circulating ECs in the serum of patients with severe hHcy or after an oral dose of methionine (100mg/kg), suggesting EC death in response to Hcy (Hladovec *et al.*, 1997), Outinen *et al* (1999) used relatively high concentrations of Hcy (≥ 10mM) in their study and that did not affect cell viability. They concluded that cultured EC from normal individuals are relatively resistant to high doses of Hcy. Although less than 1% of exogenous Hcy which was added to the media, was taken up intracellularly (Outinen *et al.*, 1999; Stamler *et al.*, 1993), even this has been shown to be enough to damage the cells (Cai *et al.*, 2000; Ji and Kaplowitz, 2004; Kokame *et al.*, 1996) and those studies suggested that intracellular Hcy is the main agent responsible for Hcy effects.

It was believed previously that most Hcy biological effects could be mimicked by cysteine which has a higher susceptibility to thiol auto-oxidation (D'Angelo and Selhub, 1997), and its normal concentration in plasma 20-25 fold higher than that of Hcy. However,

Wang *et al* (1997) was the first study to demonstrate that detrimental biological effects are specific to Hcy and that Cys does not have similar effects and that has been confirmed by later studies (Carmel and Jacobsen, 2001; Jacobsen, 2000; Sengupta *et al.*, 2001) who found that EC incubation with cysteine has no detrimental effects on EC and cysteine should not be considered as a risk factor in atherosclerosis development. However, Hcy-thiol group can perform disulfide exchange with cysteine residues in proteins. This S-thiolation by Hcy will alter the function of different proteins which are critical for maintaining vascular function like albumin, fibronectin, transthyretin, factor V, and Annexin II (reviewed by Lentz (2005)).

To summarize, Hcy appears to work through a variety of different mechanisms leading to EC dysfunction and thus initiating atherosclerosis plaque formation. However, the signalling pathway involved in mediating Hcy-induced effects in EC is still unclear.

1.2. JNK ROLE IN HCY-MEDIATED EC ACTIVATION

1.2.1. Functional effects of cellular JNK activation

JNK is a member of the MAP kinase family as already described in section 1.1.1.2 and is involved in pivotal cellular functions like apoptosis (Tournier *et al.*, 2000; Xia *et al.*, 1995), oncogenic transformation (Raitano *et al.*, 1995), cell proliferation (Xu *et al.*, 1996), EC activation and the development of atherosclerosis (Sumara *et al.*, 2005). Several studies have been reviewed by Barr and Bogoyevitch (2001) and suggested that JNK might play a role in both cell death and in cell survival. It has been shown in PC12 neuronal-like cell line to be critical for the initiation of cell death following cellular insult (Xia *et al.*, 1995). Conversely, it has been proposed that JNK might act to enhance cell survival following exposure to cell stress like in TNF-α-induced apoptosis (Roulston *et al.*, 1998). Therefore, it could be concluded that the JNK pathway might mediate different consequences depending on cell type, stimuli and environmental circumstances (Hoshino *et al.*, 2005). TNF-α is the best characterised JNK activator requiring TRAFs and IRE1s (Urano *et al.*, 2000). TRAFs are TNF receptor-activated factors and their effector function depends on the integrity of their NH2-terminal domain. IRE1s are ER-resident transmembrane kinases that function as ER stress sensor proteins and they sense stress through their conserved luminal domain and both of TRAFs and IREs are required for stress-induced JNK activation such as in the case of Hcy-induced JNK activation (Zhang *et al.*, 2001b).

Stress, like malfolded proteins or exposure to TNF-α, induces oligomerization and activation of inositol auxotrophy (IRE1) (Shamu and Walter, 1996; Urano *et al.*, 2000) and that could lead to clustering of TRAF2 which is bound to the COOH-terminal cytoplasmic portion of IRE1. Clustering of TRAF2 to the cytosolic side of the TNF receptor (Reinhard

et al., 1997) then activates proximal components of the JNK kinase cascade which in turn leads to JNK activation (Figure 6). Therefore, it could be said that JNK activation in response to ER stress might occur through a similar pathway to that activated by TNF-α (Urano *et al.*, 2000). Zhang *et al* (2001b) demonstrated that Hcy induces JNK activation downstream of IRE-1 and TRAF2 since inhibition of IRE-1 or TRAF-2 reversed Hcy effects on JNK activation. Activation of MAPK family members, including JNK, leads to activation of transcription factors which can result in immediate gene transcription of important cellular proteins and cytokines as well as MAP kinase phosphatase (MKPs) (Farooq and Zhou, 2004). MKP are a family of protein phosphatases that inactivate MAPKs through dephosphorylation of threonine and tyrosine residues within the active loop and thus work as a negative feedback mechanism for MAPK activity (Ralph and Morand, 2008). Those proteins have two distinct phospho-binding sites for the binding of p-tyrosine and p-threonine within the MAPK motif.

The effects of Hcy-induced ER stress and the subsequent activation of the JNK pathway

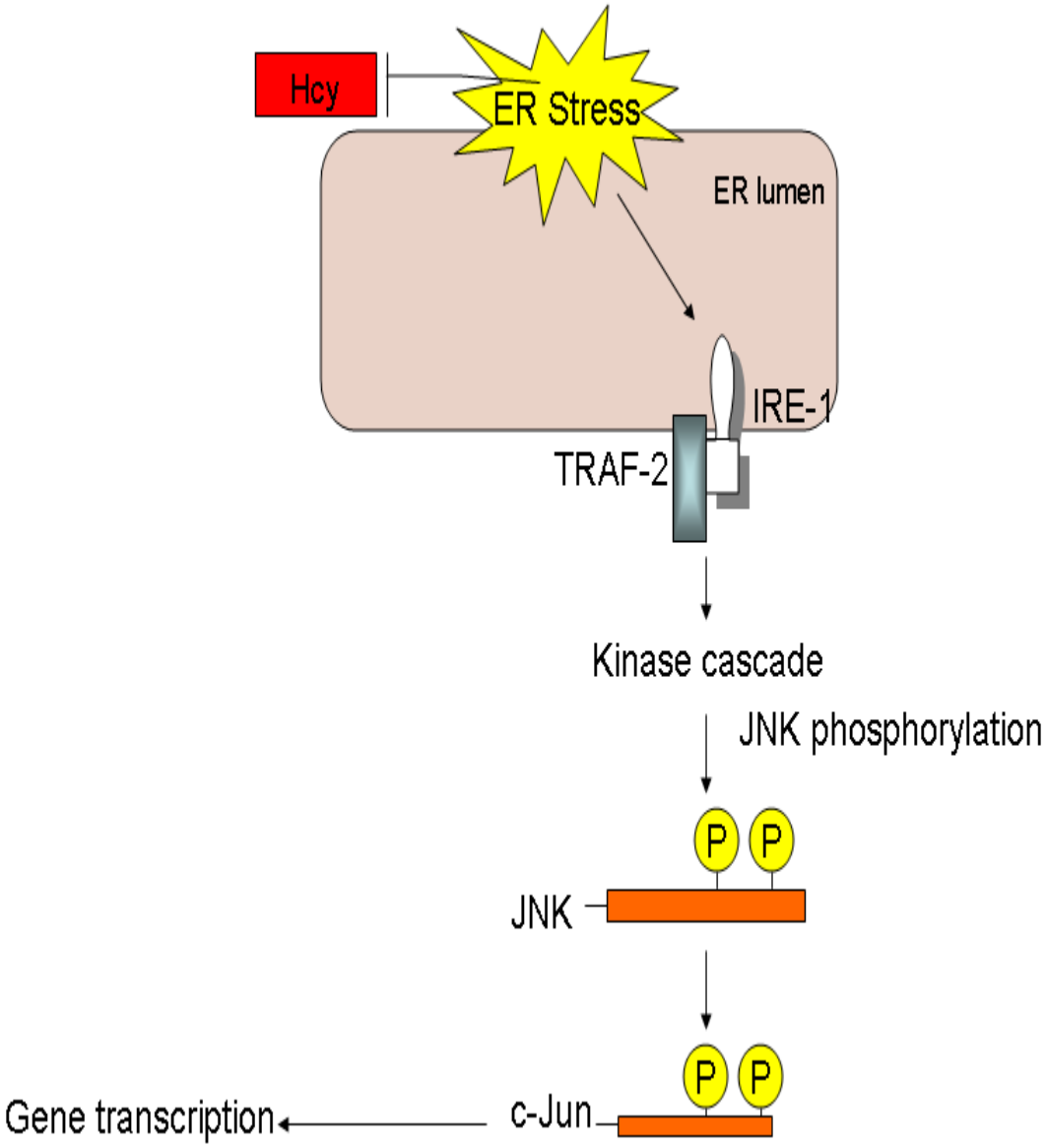


Figure 6: Hcy induces ER stress which will be sensed by IRE-1, an ER stress sensor, and initiates a cascade of events including kinase cascade activation. That leads to activation of JNK and its downstream substrate c-Jun. Activated c-Jun has binding sites on the promoter of several genes and thus induces their expression.

1.2.2. JNK in atherosclerosis

Different studies have shown JNK to be highly activated in atherosclerotic lesions in human (Nishio *et al.*, 2001) and rabbits (Metzler *et al.*, 2000). Furthermore, $JNK2^{-/-}$ mice were shown to have reduced atherosclerosis (Ricci *et al.*, 2004). JNK was also shown to mediate the up-regulation of different CAM in response to diverse stimuli (as explained in Chapter 2) and therefore mediating inflammatory responses. Collectively, that implicates JNK involvement in the atherosclerotic process. Atherosclerotic plaques can rupture and lead to further complications like coagulation and thrombosis (Lusis, 2000). Various proteases are involved in the degradation of the plaque including collagenases and matrix metalloproteins (MMPs). MMP9 is highly expressed in atherosclerotic plaques and JNK has been shown to be involved in the expression of MMP9 (Crowe *et al.*, 2001). Therefore, in addition to being involved in the early stages of the formation of the plaque through mediating CAM expression, JNK is also involved in the late stages of atherosclerotic plaque rupture (reviewed by Sumara *et al* (2005)).

1.2.3. JNK and Hcy-induced endothelial cell dysfunction

Hcy has been shown to induce JNK activation in different cell types and that led to different consequences such as cardiomyocyte (Levrand *et al.*, 2007) or VSMC apoptosis (Yuan *et al.*, 2007), up-regulation of different proinflammatory mediators like IL-8 and MCP-1 in cultured human whole blood (Zeng *et al.*, 2005) and cell adhesion molecules (Koga *et al.*, 2002; Postea *et al.*, 2006; Pruefer *et al.*, 1999).

The involvement of the JNK signalling pathway in Hcy-mediated activation of EC has been investigated in several studies. Acute exposure to Hcy was suggested to exert a cell type-specific inhibition of AP-1, downstream of JNK signalling pathway (Suzuki *et al.*,

2000). Other studies showed that Hcy caused activation of JNK and induced apoptosis in HUVEC (Dong *et al.*, 2005; Zhang *et al.*, 2001b). Cai's group also found that acute treatment for 8h with 3mM Hcy induced JNK phosphorylation in HUVEC (Cai *et al.*, 2000). Although it has been demonstrated that Hcy induces JNK activation, different mechanisms have been suggested and are still under investigation. Some investigators believe that ROS mediate the Hcy-induced activation of JNK in VSMC (Yuan *et al.*, 2007). However, several studies reviewed by Lawrence de Koning *et al* (2003) suggested that Hcy interferes with disulphide bond formation in ER and thus induces ER stress which will trigger the kinase cascade leading to JNK actication (as previously shown in Figure 6).

1.2.4. JNK as a target for therapeutic approaches

A recent study gave evidence that knocking out JNK from animals provides some therapeutic advantages (Bogoyevitch, 2005). Furthermore, $JNK2^{-/-}$ mice were shown to have reduced atherosclerosis (Ricci *et al.*, 2004). SP600125, (anthra[1,9-cd] pyrazol-6 (2H)-one), is a reversible, ATP-competitive inhibitor of JNK and has been shown to exhibit selectivity of > 20-fold relative to other tested kinases and an IC50 value of 40, 40, 90 nM for JNK1, JNK2 and JNK3 respectively (Bennett *et al.*, 2001). SP600125 was found to inhibit JNK activity in mice for a short period (4 weeks) and to efficiently inhibit atherosclerosis (Ricci *et al.*, 2004). The ATP-binding pockets of the JNK molecule provide an optimal binding environment for SP600125. However, in p38, which is not inhibited by SP600125, ATP-binding pockets appear larger and unable to provide suitable conditions for hydrophobic binding of SP600125 (all reviewed by Bogoyevitch (2005)).

On the other hand, some other papers provided evidence that the specificity of SP600125 inhibitory effects towards JNK is still under debate (Bain *et al.*, 2003). They

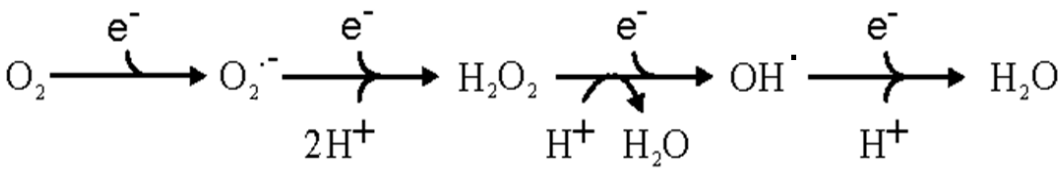
provided evidence that 13 more protein kinases are inhibited to the same or greater extent by SP600125. However, none of these kinases is thought to be involved in mediating the effects of proinflammatory cytokines or other stresses that lead to expression of different CAMs.

To summarise, the JNK signalling pathway is highly suspected to mediate Hcy-induced EC damage and targeting this pathway might provide a potential therapeutic approach to protect from atherosclerosis. However, the mechanism through which Hcy induces activation of JNK pathway and the subsequent effects under flow is still in debate.

1.3. THE ROLE OF OXIDATIVE STRESS IN HCY-MEDIATED EC ACTIVATION

1.3.1. Reactive oxygen species in cell function and dysfunction

Oxygen is an essential molecule for aerobic metabolism and one electron reduction of oxygen produces the highly reactive free radical superoxide anion ($O_2^{\bullet -}$) (Sies, 1997). Superoxide production is spontaneous in the electron-rich aerobic environment in the vicinity of the inner mitochondrial membrane with the respiratory chain, however, it cannot penetrate lipid membranes and therefore is trapped where it was generated (Nordberg and Arner, 2001). The reduction of molecular oxygen via 1-electron transfer, produces superoxide anion and other radicals including OH-radicals and hydrogen peroxide which all belong to a family called the reactive oxygen species (ROS) (Nordberg and Arner, 2001):



All of these molecules are known to be highly reactive due to the presence of unpaired valence shell electrons. ROS level is maintained by the balance between ROS-generating mechanisms and the efficiency of antioxidant enzymes (explained in Figure 7). However, generation of high levels of ROS and/or impaired defective function of antioxidant defence system results in a cellular condition called oxidative stress (Sies, 1997).

The enzyme systems involved in the production and elimination of reactive oxygen species

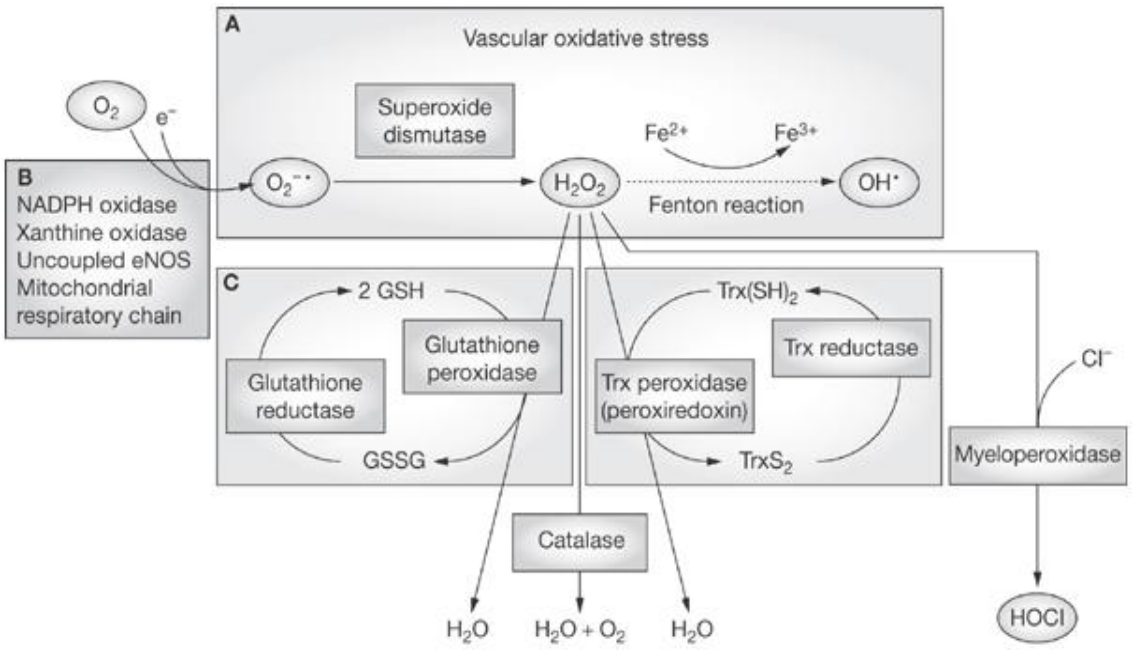


Figure 7: (A) Overproduction of reactive oxygen species results in oxidative stress and superoxide dismutase catalyzes dismutation of superoxide into oxygen and hydrogen peroxide. (B) Enzymes which might be responsible for producing ROS. (C) Hydrogen peroxide can be detoxified via different enzymes: GSH: monomeric glutathione; GSSG: reduced glutathione; Trx: thioredoxin. Adapted from Forstermann (2008).

ROS have been shown to exist in biological cells and tissues at low but measurable concentrations (Droge, 2002) and different cell types like SMC, fibroblasts and EC have been shown to produce ROS at relatively low levels in response to extracellular signals or stimuli (reviewed by Kunsch and Medford (1999)). Different redox-sensitive transcriptional pathways have been described and ROS were suggested to play a role as second messengers to mediate signal transduction pathways which lead to gene expression and posttranslational modification of proteins (Kunsch and Medford, 1999; Zhang and Gutterman, 2007). They are also involved in regulating oxygen sensing, vascular tone, cell

growth and apoptosis (Droge, 2002). Furthermore, ROS play a vital role in killing invading bacteria, thus are a defence mechanism against infection (Nordberg and Arner, 2001).

On the other hand, once ROS are produced in excess leading to oxidative stress conditions, they can readily react with most biomolecules, starting a chain of free radical formation. They also have detrimental effects that trigger alternative pathways and the transcription of several genes which leads to an altered expression of cytokines, chemokines and adhesion molecules (Papatheodorou and Weiss, 2007) and causes cell damage and toxicity (Kyaw *et al.*, 2004). ROS can also induce DNA damage and chemical modification of DNA (Nordberg and Arner, 2001). They induce oxidation of low-density lipoproteins (LDL) which are then taken up by phagocytes forming foam cells in the earliest stage of atherosclerotic plaque formation (Lusis, 2000).

The major sources of ROS are NADPH oxidase, xanthine oxidase, the enzymes of mitochondrial respiratory chain and uncoupled eNOS (Forstermann, 2008). NADPH oxidases are multicomponent enzymes that work in the membrane of vascular EC, SMC and fibroblasts and several studies have provided evidence of increased activity of the NADPH oxidase in atherosclerotic arteries (Sorescu *et al.*, 2002). Furthermore, several studies reviewed by Forstermann (2008) have used genetic disruption of NADPH oxidase subunits and confirmed the role of NADPH oxidase-derived ROS in hypertension and atherosclerosis. Xanthine oxidase donates electrons to molecular oxygen and thus produces superoxide and hydrogen peroxide (Figure 7) (Forstermann, 2008). It is found membrane-associated on EC and also found to be responsible for vascular oxidative reactions leading to endothelial dysfunction (Ullrich and Bachschmid, 2000). Two more sources of ROS have been also defined, eNOS uncoupling (to be discussed in detail in Chapter 5) and the

mitochondrial enzymes which are believed to reduce 1% of the oxygen that is consumed by mitochondria, forming superoxide (Forstermann, 2008).

Several enzymes have been identified which protect from oxidative stress (reviewed by Forstermann (2008) and Nordberg and Arner (2001)) and illustrated in Figure 7 including: superoxide dismutase (SOD) which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Catalases which catalyze the dismutation of hydrogen peroxide to water and molecular oxygen and also bind NADPH, which protects the enzyme from inactivation and increases its efficiency. Glutathione peroxidase (GPx) which reduces lipid hydroperoxides to their alcohol and reduces free hydrogen peroxide into water. Some studies indicate that GPx works as antioxidant under physiological conditions (Jones *et al.*, 1981), while others believe that the enzyme is important only in events of oxidative stress (Kelner and Bagnell, 1990).

1.3.2. Oxidative stress and vascular endothelium

Oxidative stress has been suggested to induce EC activation and dysfunction by altering different pathways (Cai and Harrison, 2000). The transcription factors NF-κB and AP-1 have been shown to be redox sensitive and to be regulated by intracellular redox state (Meyer *et al.*, 1994) and these transcription factors are involved in the regulation of many genes such as ICAM-1, VCAM-1, E-selectin and MCP-1. All of the previous genes encode for different receptors and chemokines which play important roles in the initiation of healthy acute inflammation, but also atherosclerosis (Forstermann, 2008; Kunsch and Medford, 1999). Oxidative stress also has a very well established relationship with hypertension as angiotensin II signalling leads to NADPH oxidase up-regulation and activation resulting in oxidative stress (Zalba *et al.*, 2001). Furthermore, ROS have been

shown to mediate activation of MAPK family members like p38 in cultured vascular SMC (Ushio-Fukai *et al.,* 1998) and JNK in fibroblasts (Kamata *et al.,* 2005) and both pathways are involved in the activation of several genes encoding proinflammatory cytokines and CAM (Chang and Karin, 2001). The relaxing factor NO also presents another target for ROS as under oxidative stress conditions, $O_2^{\bullet -}$ might interact with NO decreasing its bioavailability and forming the very potent oxidant peroxynitrite which leads to impairment of endothelium-dependent vasorelaxation and activation of EC (Cai and Harrison, 2000). Lipid peroxidation is another target for ROS and, for example, oxidized LDL is very cytotoxic for EC and induces inflammatory cell recruitment to the endothelium; it also interacts with NO and decreases its bioavailability (Chin *et al.,* 1992). All of the previously described ROS-altered pathways and genes work together to activate EC and promote the initiation of atherosclerosis.

1.3.3. Homocysteine and oxidative stress-mediated vascular dysfunction

Hcy interferes with redox signalling in EC and that contributes, at least in part, to Hcy-induced impairment of normal EC functions which leads to development of atherosclerosis (Papatheodorou and Weiss, 2007). Methionine loading induced homocysteinemia in healthy donors leading to EC damage (Bellamy *et al.*, 1998; Chambers *et al.,* 1998; Kanani *et al.,* 1999). The previous groups characterized EC damage by reporting impaired brachial artery flow-mediated dilatation which is known to be mediated by endothelial NO and that suggested impaired endothelial NO activity in healthy individuals during acute hHcy. These effects were reversed by administering vitamin C, a ROS scavenger (Kanani *et al.*, 1999), suggesting that Hcy effects were mediated by ROS. Different mechanisms have been suggested to explain how Hcy may increase ROS in

vascular cells and tissues (Figure 8). Auto-oxidation of the thiol group in Hcy will generate superoxide anions ($O_2^{\bullet-}$) (Misra, 1974).

$$2RSH + O_2 \rightarrow RSSR + [O_2^{\bullet-}] \rightarrow H_2O_2$$

RSH: Hcy and RSSR: homocystine

Hcy was suggested also to disturb eNOS efficiency by reduction of its cofactor tetrahydrobiopterin (BH_4) leading to eNOS uncoupling which will start generating superoxide anions instead of NO (Heydrick *et al.*, 2004) (this theory will be discussed in more detail in Chapter 5). Hcy has been suggested also to inhibit or alter the efficiency of antioxidant enzymes in BAEC like cellular haem oxygenase 1 (Sawle *et al.*, 2001) or cellular glutathione peroxidase (Handy *et al.*, 2005). Hcy also has been shown to decrease the activity and steady-state mRNA levels of glutathione peroxidase in BAEC (Loscalzo, 1996; Upchurch *et al.*, 1997) and to suppress the expression of glutathione peroxidase in HUVEC (Outinen *et al.*, 1999). Furthermore, the presence of copper ions was suggested to be essential for Hcy to cause oxidative cell injury and suppress glutathione peroxidase and superoxide dismutase in HUVEC (Zhang *et al.*, 2001a). Collectively, Hcy promotes endothelial dysfunction by indirectly impairing the endothelial antioxidant defence mechanism and inducing oxidative stress.

Dong *et a*l found that acute treatment with Hcy induced ROS generation in HUVEC which they suggested to be mediated by NADPH oxidase and/or JNK (Dong *et al.*, 2005). Several other studies also reported that Hcy induces NADPH oxidase activity. It was suggested this was by activating de novo synthesis of ceramide which stimulates Rac GTPase activity in rat mesangial cells (Yi et al., 2007; Yi et al., 2004). Another group

(Edirimanne *et al.*, 2007) showed in rats that overloading with methionine markedly induced NADPH oxidase activity, particularly $p22^{phox}$ which was responsible for increased production of superoxide anion, as apocynin (NADPH oxidase inhibitor) managed to reverse these effects. Furthermore, administration of apocynine restored endothelium-dependent relaxation in the aortas of hHcy rats (Edirimanne *et al.*, 2007). Hcy was also shown to induce stimulation of NADPH oxidase $p47^{phox}$ and $p67^{phox}$ subunits in monocytes (Siow *et al.*, 2006) and neutrophils (Alvarez-Maqueda *et al.*, 2004). Data from these studies indicate the importance of NADPH oxidase in mediating Hcy-induced oxidative stress in different vascular cells.

Extracellular superoxide dismutase (SOD) from the endothelial surface was suggested to be a target for Hcy interference (Wilcken *et al.*, 2000). SOD has a Cys residue at the active site which might form disulfide bonds with the free thiol group of Hcy and thus inhibit the enzyme activity (Atmaca, 2004). Interestingly enough, one study found that hHcy was associated with elevated levels of extracellular SOD suggesting a protective role of extracellular SOD against the oxidant effects associated with hHcy (Wilcken *et al.*, 2000). Furthermore, Hcy was shown to activate NADPH oxidase which is the main source of $O_2^{\bullet -}$ in vascular cells (Alvarez-Maqueda *et al.*, 2004; Dong *et al.*, 2005; Ungvari *et al.*, 2003).

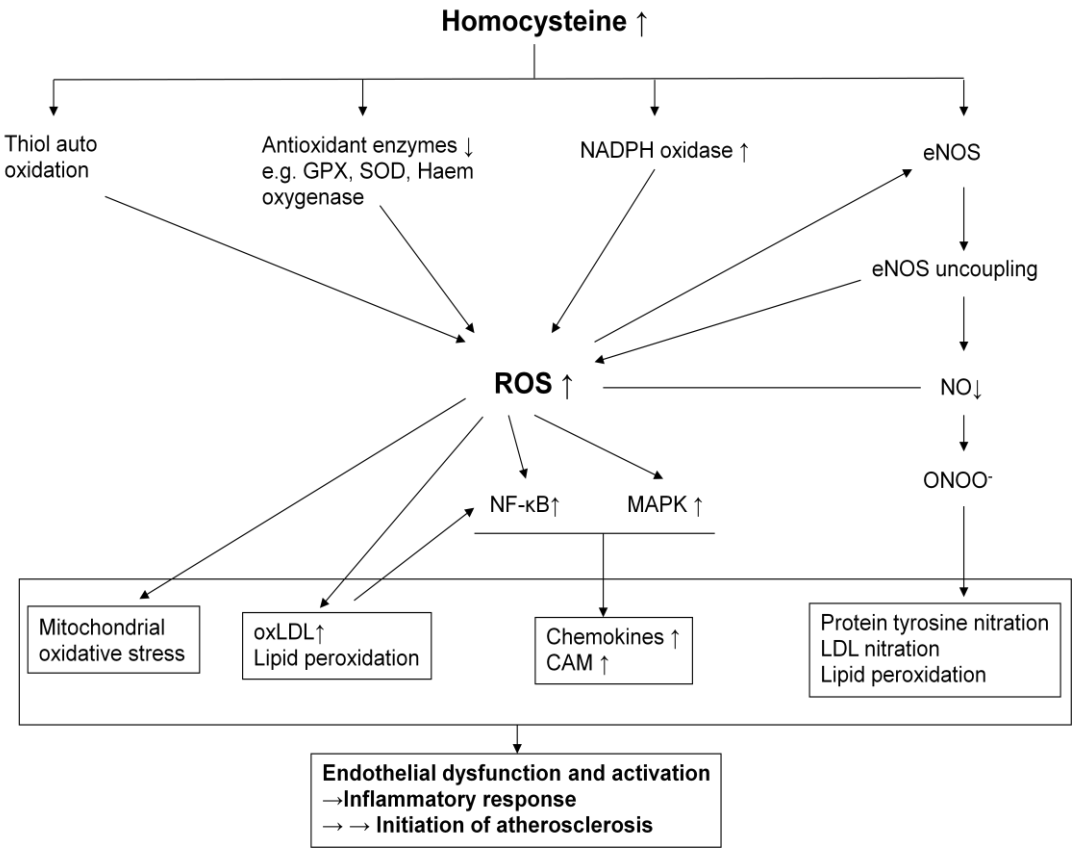


Figure 8: Pathways through which Hcy induces generation of ROS and the consequences of these ROS. Expanded from Papatheodorou and Weiss (2007).

Some studies (Ballinger *et al.*, 2002; Ide *et al.*, 2001) have demonstrated that increased generation of ROS is associated with mitochondrial damage on different levels: increased lipid peroxidation in mitochondria, decreased mitochondrial DNA copy number, decreased mitochondrial RNA transcripts and reduced oxidative capacity due to low enzyme activity. Mitochondrial DNA can easily be damaged by ROS and the mutated mitochondrial DNA accelerates ageing and the development of CVD (Ballinger *et al.*, 2002; Lim *et al.*, 2001). Furthermore, Hcy-induced ROS act as upstream factors for mitochondrial membrane depolarization (Tyagi *et al.*, 2006). This group found, in rat heart microvascular EC, that Hcy-induced ROS generation causes mitochondrial membrane

depolarization, cytochrome-c release and activation of caspase-9 therefore leading to EC apoptosis (Tyagi *et al.*, 2006). It is highly likely that all of these mechanisms, or at least some of them, work synergistically to mediate Hcy-induced oxidative stress in EC. However, the effects of chronic Hcy-induced oxidative stress have not yet been illustrated and also the mechanism by which ROS mediate Hcy atherogenic effects is not fully addressed.

1.3.4. The efficiency of antioxidant treatment

Antioxidants could be divided into two major groups, enzymatic (explained earlier in section 1.3.1) and non enzymatic antioxidants. Non-enzymatic antioxidants have been widely used in different *in vivo* and *in vitro* studies like vitamin C (Yamashita *et al.*, 2005), vitamin E (Kerkeni *et al.*, 2006), glutathione and NAC (Takacs *et al.*, 2001) to overcome oxidative stress and retrieve normal cell conditions. Two different non-enzymatic antioxidants, vitamin C and NAC, were used in this thesis to confirm that any effects are due to ROS scavenging and not to any interactions with Hcy.

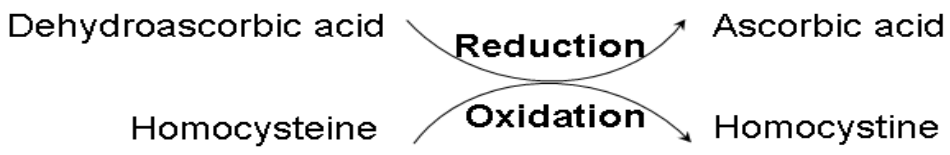
1.3.4.1. N-acetyl Cysteine

N-acetyl Cysteine (NAC) could be derived from food or produced within the human body from Cys and is rapidly metabolized to intracellular glutathione which acts as a powerful antioxidant in the body (Atmaca, 2004). Synthesis of the antioxidant glutathione requires three amino acids, glutamic acid, glycine and Cys. Both glutamic acid and glycine are plentiful in cells and therefore the availability of Cys controls glutathione production (Atmaca, 2004). NAC enters the cell and is readily hydrolyzed to Cys thus inducing the production of glutathione (Sochman, 2002). NAC was shown to inhibit NF-κB activation in HUVEC cultured with plasma from women with severe preeclampsia (Takacs *et al.*, 2001).

The effect was shown to be through scavenging ROS which are known to activate NF-κB and also block the expression of ICAM-1 mediated by NF-κB. NAC was also shown to inhibit TNF-α-induced cytokine expression like IL-8 and MCP-1 in human synovial cells (Sato *et al.*, 1996). Furthermore, NAC was shown to block TNF-α-induced JNK activation in a transformed EC cell line (ECV-304) (Gu *et al.*, 2002) and also could reduce peroxynitrite formation by direct reaction of thiol group in NAC and NO to produce nitrothiol compounds (Atmaca, 2004). NAC was also suggested to form disulphide bonds with thiol-containing molecules like Hcy, increasing their renal clearance efficiency (Ventura *et al.*, 1999). Ventura's study showed that single intravenous administration of NAC to healthy subjects induces an efficient and rapid clearance of plasma thiols like Hcy and therefore, decreases plasma levels of the reduced form of Hcy which has the deleterious consequences.

1.3.4.2. Vitamin C

Vitamin C primarily exists as ascorbic acid (a reduced form) in human plasma and is the most effective water soluble antioxidant in human plasma (Forstermann, 2008; Yamashita *et al.*, 2005). In HUVEC, Hcy was shown to be involved in the reduction of dehydroascorbic acid, the oxidized form of ascorbic acid, to ascorbic acid and in the meantime homocysteine is oxidized to homocystine (Park, 2001):



The authors mixed the two compounds in HEPES/phosphate buffer and the interaction happened very quickly within 3min. They believe that this interaction reduces

the deleterious effects of homocysteine (Park, 2001). Park believes that homocysteine and homocystine are in a steady state equilibrium and the reduction of dehydroascorbic acid to ascorbic acid can shift this equilibrium toward producing homocystine which was thought to be not as harmful as Hcy (Cai *et al.*, 2000). However, another study found that homocystine has the same deleterious effects as Hcy (Heydrick *et al.*, 2004) which contradicts Park's findings and indicates that vitamin C effects are due to scavenging ROS and not due to any effects on a Hcy/homocystine equilibrium. Several reports illustrated that acute administration of vitamin C reduced oxidative stress and reversed Hcy coronary effects in patients with experimentally induced hHcy (Yamashita *et al.*, 2005) and other groups found that vitamin C administration (1g per day) blocked the EC dysfunction which was induced by oral methionine load (100mg/kg) in human subjects (Chambers *et al.*, 1999; Nappo *et al.*, 1999). Furthermore, vitamin C and vitamin E reduced Hcy-induced apoptosis in HUVEC by 45% (Kerkeni *et al.*, 2006).

Vitamin C protects NO levels by scavenging ROS or preventing oxidized LDL formation and thus preserving NO bioavailability. Some other studies suggest that vitamin C enhances the availability and stability of BH_4 or increases its affinity to eNOS and therefore vitamin C restores EC function by restoring BH_4 and thus restoring eNOS activity rather than just scavenging ROS (Heller *et al.*, 2001). However, other antioxidants like vitamin E can become a radical itself and that could limit its efficiency as an antioxidant *in vivo* (Forstermann, 2008).

1.3.4.3. Antioxidants in clinical trials

All the *in vivo* and *in vitro* studies described above demonstrated the efficiency of antioxidants in reversing endothelial damage and preventing development of

atherosclerosis. Furthermore, some studies found inverse relationship between CVD and plasma levels of vitamins E and C (Gey *et al.,* 1991; Su *et al.,* 1998). These encouraging findings lead to different randomised clinical trials that have been conducted using antioxidants to study whether scavenging ROS might have any therapeutic benefits in patients with high risk of CVD. However, no beneficial effect was noticed from vitamin C supplementation in terms of reducing the risk of coronary diseases or cardiovascular morbidity or mortality (Paolini *et al.,* 2003). Furthermore, randomized trials reviewed by Stanner *et al* (2004) have failed to show any consistent benefit from the use of antioxidant supplements on CVD where they used single antioxidant nutrients given at relatively high doses. Forstermann in his review (Forstermann, 2008) summarizes some potential reasons for the lack of effects of antioxidants in clinical trials. Inappropriate dosing might lead to insufficient supplement of the antioxidant and therefore they won't be able to overcome the oxidative stress situation. Furthermore, Forstermann explains that vitamin C and vitamin E interaction with superoxide happens at a rate constant of 4-5 times of magnitude lower than the rate constant of the reaction between NO and superoxide. Furthermore, those antioxidants have no effects on hydrogen peroxide or hypochlorous acid which both might be involved in vascular damage (Forstermann, 2008). More studies are needed to explain the lack of efficiency of antioxidants in clinical trials and to identify possible combinations of these antioxidants which might help to improve the efficiency of the treatment.

To summarize, it is clear that chronic Hcy induces oxidative stress in EC which was responsible for activating JNK pathway and all the subsequent effects under static and flow conditions. However, the source of Hcy-induced ROS production is still unclear.

1.4. THE ROLE OF NITRIC OXIDE AND NITRIC OXIDE SYNTHASE IN HCY-MEDIATED ATHEROGENIC EFFECTS

1.4.1. Nitric oxide biology

Nitric oxide is one of the smallest molecules found in nature consisting of one atom of nitrogen and one atom of oxygen which results in unpaired electron making NO a radical (Lincoln *et al.*, 1997). NO has been studied intensively as a key factor in the functions of EC and also has antiatherosclerotic properties including SMC relaxation reducing vascular tone, inhibition of platelet aggregation and inhibition of leukocyte adhesion (reviewed by Naseem (2005)). It has been also reported that NO can inhibit LDL oxidation by reacting with lipid peroxyl radicals (Rubbo *et al.*, 1994). Furthermore, NO has been studied as a potential therapeutic agent and NO donors like nitroglycerine are being used in protection from myocardial infarction and other CVD complications (Gryglewski and Minuz, 2001).

In biological systems, NO has a very short half-life of about 30s and, as it is uncharged, it diffuses within cells and across membranes. NO is synthesized from the amino acid L-arginine in a reaction which is mediated by the enzyme nitric oxide synthase (NOS). In addition to L-arginine and NOS, cofactors required for NO synthesis are: calcium/calmodulin, BH_4, haem, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Butler and Nicholson, 2003) (Figure 9). A lack of any of these factors will negatively affect the efficiency of NO production. NOS is synthesized as a monomer and includes a reductase domain and oxygenase domain that carries a haem group. Monomers can bind neither the substrate L-arginine nor the cofactor BH_4 and therefore cannot catalyze NO production. The haem allows the active NOS dimer to be formed and plays a major role in the interaction between the reductase and oxygenase

domains and the electron flow from the flavin to the haem of the opposite monomer. In the presence of sufficient L-arginine and BH_4, NOS dimers couple their haem and start synthesizing NO and the byproduct L- citrulline (Forstermann and Munzel, 2006) (Figure 9). Three different isoforms of NOS have been identified and are named in accordance to the location where they were first found, endothelial NOS (eNOS) in endothelial cells, neuronal NOS (nNOS) in the brain and the inducible form (iNOS) in macrophages. These isoforms are also known as NOS-3, NOS-1 and NOS-2 respectively (Alderton *et al.*, 2001).

Reactive nitrogen species (RNS) are nitrogenous products includes in addition to NO, compounds like nitrogen dioxide (NO_2) which are produced by NOS. These products have chemical and biological properties and can cause nitrosation and nitration of different molecules leading to nitrosative stress. Furthermore, ROS and RNS can work synergistically and produce compounds which exert both oxidant and nitrosative activities like $ONOO^-$ (reviewed by Shen and Liu (2006)).

The structure of eNOS and the production of NO

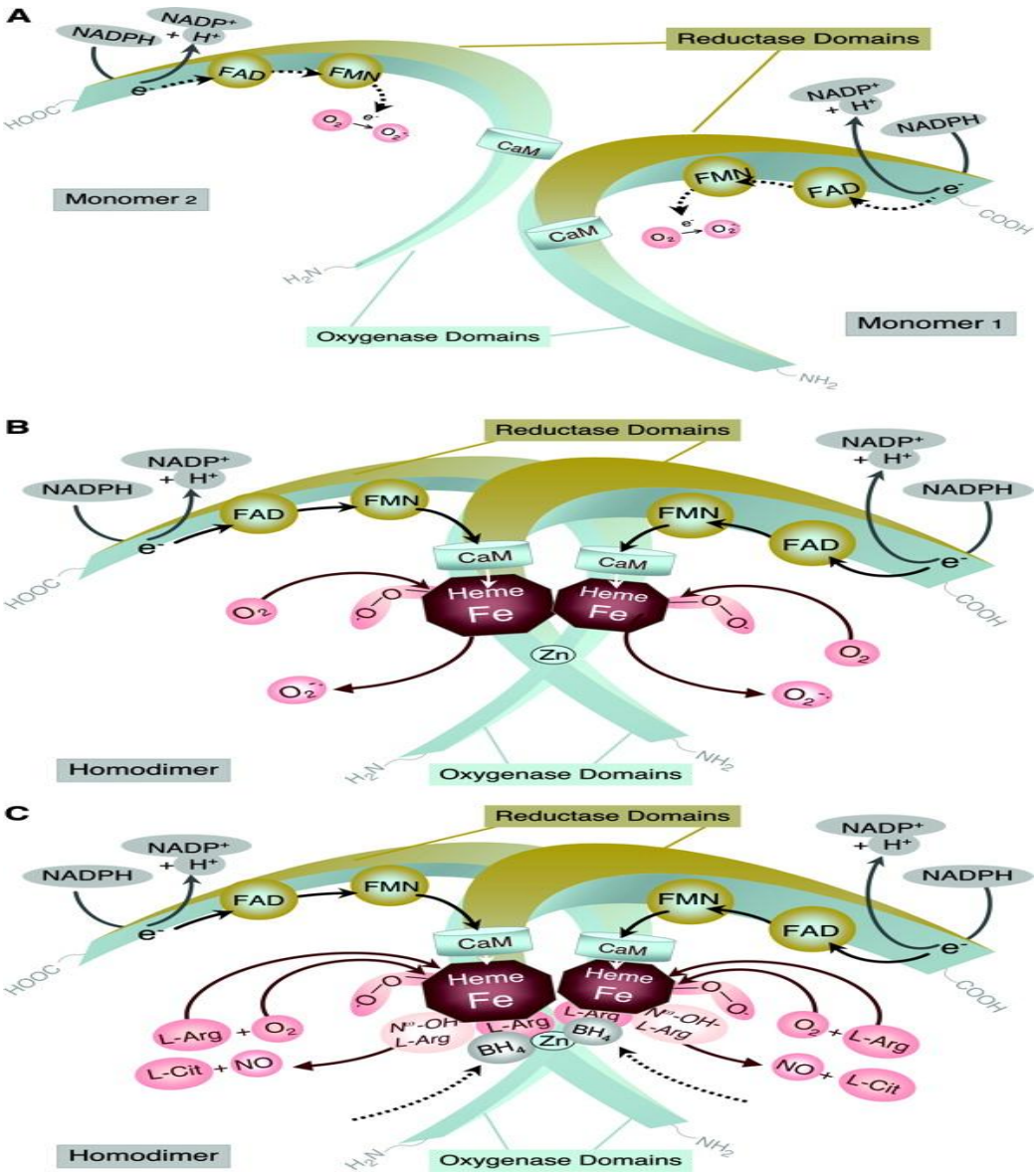


Figure 9: eNOS structure and the catalysis of NO production. (A): monomeric structure. (B): dimeric structure, the haem allows the active NOS dimer to be formed. Haem plays a major role in the interaction between reductase and oxygenase domains and the electron flow from the flavin to the haem of the opposite monomer. Once sufficient L-arginine and BH_4 are present, NOS dimers couple their haem and start synthesizing NO and the byproduct L-citrulline (C). Adapted from Forstermann and Munzel (2006).

1.4.2. Nitric oxide and endothelial cells

NO can be synthesized by different cell types where it can control different physiological processes and this thesis will focus on the NO produced by EC. eNOS is the main enzyme in EC responsible for NO production. However, there is some evidence that iNOS can be expressed in EC and induces NO generation in response to some stimuli (Kroll and Waltenberger, 1998). NO action is via two different mechanisms, the first one happens via activating soluble guanylyl cyclase (sGC) and the production of cGMP (Schlossmann *et al.*, 2003), while the second mechanism depends on NO ability to cause nitrosylation of some target proteins altering their efficiency (Lowenstein, 2007).

1.4.2.1. cGMP-dependent effects

NO binds to the iron of the haem at the active site of sGC inducing its activation to generate the cGMP. cGMP activates different proteins like cGMP-dependent protein kinases which regulates vascular tone and platelet aggregation. These kinases are thought to reduce intracellular calcium release and lower the calcium sensitivity of the contractile apparatus leading to vasorelaxation in SMC (reviewed by Schlossmann *et al* (2003)).

1.4.2.2. Nitrosylation

Nitrosylation has emerged as a posttranslational modification of signalling proteins (Derakhshan *et al.*, 2007). It happens when the free sulfur atom from Cys or Hcy residues reacts with NO resulting in S-nitrosothiol moieties (Gaston *et al.*, 2003). In proteins which are known to undergo significant nitrosylation, it is noted that only one out of many Cys residues undergoes a detectable level of NO addition (Derakhshan *et al.*, 2007). Several studies reviewed by Lowenstein (2007) showed that NO interferes with protein trafficking from the Golgi apparatus to the plasma membrane and from the plasma membrane to the

endosome. Some proteins show higher susceptibility for nitrosylation than others and Jaffrey's group suggested a possible explanation could be because they contain a higher number of cysteine residues (Jaffrey *et al.*, 2001). However, this group were unable to detect nitrosylation by endogenously produced NO which might be due to the fact nitrosylated proteins present at low levels compared to nitrosothiols which are formed after treatment with exogenous NO donors. The effects of nitrosylation vary between different proteins. It up-regulates Ras and the ryanodine receptor (Derakhshan *et al.*, 2007; Sun *et al.*, 2001) as well as down-regulating the activity of caspase-3 in EC (Hoffmann *et al.*, 2003), JNK in murine macrophages and microglial cells (Park *et al.*, 2000a) and NF-κB in human respiratory cells and murine macrophages (Marshall and Stamler, 2001). Some of these effects, such as down-regulating JNK and NF-κB, suggest atheroprotective effects of NO via nitrosylation and hence these effects of NO may present another way to control inflammation and thrombosis. Therefore, endothelial cellular ability to synthesize NO is key for maintaining healthy vessel tone, haemostasis and reducing leukocyte adhesion and blood pressure (Naseem, 2005).

1.4.3. Anti-inflammatory effects of NO in EC

Exposure of HUVEC to shear stress (15 dynes/cm^2, 24h) increased the overall content of S-nitrosylated proteins in a time dependent manner, reaching a maximum after 24h (Hoffmann *et al.*, 2003). Therefore, Hoffmann's group concluded that shear-stress induced production of NO which in turn induces nitrosylation of specific signaling proteins might represent an atheroprotective mechanism of shear stress. Nitrosylation effects vary between different targets and the consequences have attracted lots of attention in the past

few years as a mechanism by which NO regulates the pathways that control different cellular functions.

1.4.3.1. NO regulates CAM expression

NO has been suggested to be involved in regulating CAM expression as a way to control inflammatory responses and limit EC activation (Takahashi *et al.*, 1996). However, effects of NO on CAMs vary between different molecules and different cells. NO was shown to suppress VCAM-1 expression (Khan *et al.*, 1996; Takahashi *et al.*, 1996; Zampolli *et al.*, 2000) in cytokine-stimulated EC. Information about NO effects on ICAM-1 expression is still contradictory as NO donors were reported to inhibit ICAM-1 expression in cytokine-stimulated human EC (Spiecker *et al.*, 1998; Takahashi *et al.*, 1996), while another study found that NO donors didn't have any effects on lipopolysaccharide-induced ICAM-1 expression in HUVEC (Kosonen *et al.*, 2000). Furthermore, other studies have demonstrated that NO suppresses P-selectin expression at the transcriptional level in cultured human iliac endothelial cells (Armstead *et al.*, 1997) and *in vivo* in mice (Gauthier *et al.*, 1994). Some of these studies demonstrated that, unlike other NO cardiovascular effects discussed earlier, NO-mediated CAM down-regulation was cGMP-independent (Spiecker *et al.*, 1998; Takahashi *et al.*, 1996). Furthermore, Conran *et al* postulated that endogenously produced NO inhibits the expression of VLA-4-integrin on the neutrophil surface in a cGMP-independent manner and thus inhibits the adhesion of neutrophils to extracellular matrix components (Conran *et al.*, 2003). Furthermore, Adams *et al* showed in cultured HUVEC that L-arginine reduces monocyte adhesion to EC. This effect was associated with decreased endothelial expression of ICAM-1 and VCAM-1 (Adams *et al.*, 1997).

NF-κB is a transcription factor required for some CAM expression and its activation by cytokines such as TNF-α is thought to occur, in part, via ROS. It is believed that NO inhibits NF-κB, at least in part through scavenging and inactivating the superoxide anion, and that accounts for NO inhibitory effects on these CAMs in cytokine-stimulated EC (Conran *et al.*, 2003; Khan *et al.*, 1996). NO was also suggested to S-nitrosylate NF-κB and thus inhibit its activity which in turn leads to down-regulation of CAM expression (Marshall and Stamler, 2001). Collectively, it could be said that suppressing the expression of such CAM leads to inhibition of adhesion of neutrophils to EC and thus contributes to the prevention of the development of atherosclerotic lesions *in vivo*.

Nitrosylation of some key signalling proteins like JNK (Park *et al.*, 2000a) or transcription factors like NF-κB (Marshall and Stamler, 2001) might be responsible for the NO-mediated down-regulation of some CAM. Furthermore, certain thiol groups on EC surface are thought to contribute to normal EC:leukocyte adhesion. Those thiol groups were suggested to be nitrosylated by RNS which will inhibit their contribution to EC:leukocyte interactions and thus disturb the inflammatory response (reviewed by Hall *et al* (2000)). These effects of NO are thought to be related to its actual interaction with Cys residues in a range of proteins to form s-nitrosothiols and that the common NO downstream cGMP is not involved in this mechanism (Gaston *et al.*, 2003).

On the other hand, a study showed that both IL-1β and histamine-induced P-selectin expression in HUVEC were down-regulated by activation of sGC and therefore the production of cGMP (using BAY 41-2272) while activating sGC had no effects on IL-1β-induced E-selectin or ICAM-1 expression (Ahluwalia *et al.*, 2004). Furthermore, they reported that eNOS knockout mice showed increased leukocyte rolling and adhesion.

However, activating sGC and the subsequent production of cGMP inhibited leukocyte adhesion in the eNOS knockout mice. The anti-leukocyte effect was noticed immediately after treatment, meaning it cannot be a result of decreased selectin mRNA production and Ahluwalia's group suggested this effect was a rapid reversal of the proadhesive activity of the relevant adhesion molecule by an as yet unknown mechanism.

1.4.3.2. Weibel -Palade body (WPB) exocytosis and nitrosylation

WPB exocytosis allows delivery of their contents of P-selectin and vWF to the cell surface and this process involves a series of discrete stages (Lowenstein *et al.*, 2005). In the Golgi apparatus, the WPB granule is loaded with vWF and P-selectin before leaving the Golgi. The granule is directed by kinesin along a network of microtubules to the plasma membrane where one molecule of vesicle SNARE (v-SNARE) protein on the granule membrane interacts with two target-SNARE (t-SNARE) proteins on the plasma membrane leading to granule docking with the plasma membrane and it becoming primed for fusion. The complex of three SNARE molecules specifies which vesicle will fuse with a particular membrane (Figure 10). An influx of calcium then triggers fusion of the granule and cytoplasmic membranes, causing exocytosis. N-ethylmaleimide sensitive factor (NSF) is a protein superfamily that is involved in regulating exocytosis. NSF primes the granule for fusion, facilitates empty granule recycling, or may mediate both processes (reviewed by Lowenstein (2007) and Lowenstein *et al* (2005)). The formation of the ternary SNARE complex is a key step in vesicle trafficking, and different proteins accelerate the formation of this complex like Sec/Munc proteins, whereas NSF converts the chemical energy of ATP hydrolysis into mechanical energy to disassemble the SNARE complex (reviewed by Lowenstein (2007)). Several factors which trigger EC exocytosis were reviewed by

Lowenstein *et al* 2005 and they suggested that EC exocytosis is a final common pathway by which EC responds to injury. Interfering with WPB exocytosis has important consequences concerning P-selectin expression and thus the interactions between leukocytes and EC. Different compounds can inhibit WPB exocytosis, like H_2O_2 and NO (Lowenstein *et al.*, 2005), thus limiting, at least in part, the vascular inflammation. Lowenstein's group suggested that H_2O_2 inhibits WPB exocytosis in cultured EC by inhibiting the ATPase activity of NSF. It has been suggested by the same group and others, that NO nitrosylates NSF protein in HAEC (Matsushita *et al.*, 2003) and in platelets (Morrell *et al.*, 2005) thus inhibiting its activity and therefore preventing WPB exocytosis. Therefore, it could be concluded that NSF activity is redox sensitive and in the presence of physiological levels of oxidants, NSF controls exocytosis, therefore limiting leukocyte trafficking and the inflammatory response.

WPB exocytosis and the role of NO in regulating this process

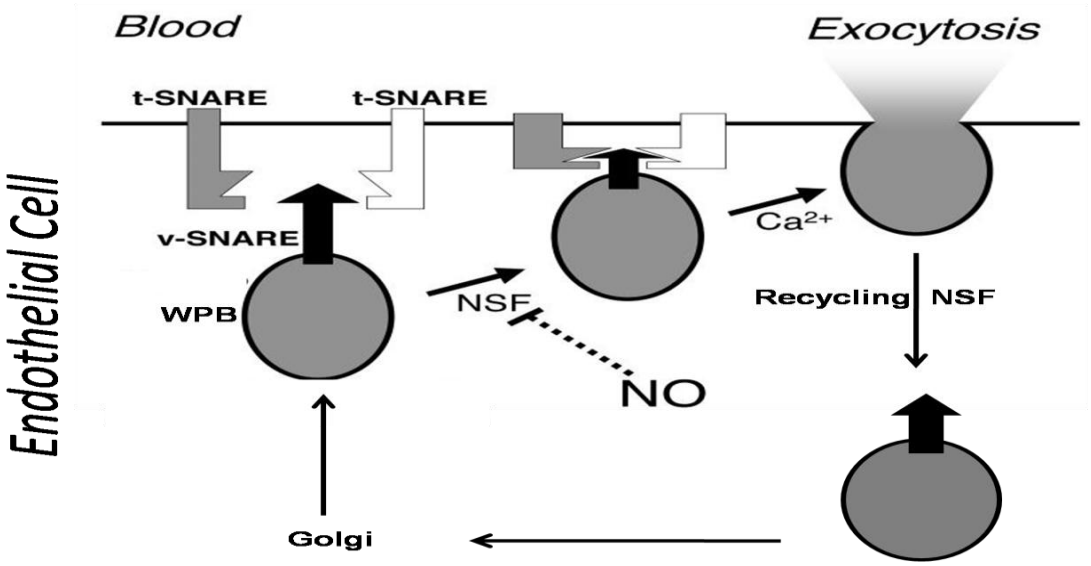


Figure 10: WPB exocytosis. Several protein superfamilies are involved in the exocytosis. NSF can be nitrosylated by NO which will inhibit their activity and therefore inhibit WPB exocytosis. Adapted from Lowenstein (2007).

1.4.3.3. JNK nitrosylation

JNK has a cysteine residue (Cys^{116} in JNK1) which is sensitive to thiol-modifying agents, like nitrosylating agents (Park *et al.*, 2000b). This cys^{116} is conserved only among the JNK subgroup of MAP kinases which might mean that NO may specifically repress JNK activity by nitrosylation (Hall *et al.*, 2000).

Some researchers believe that RNS might inhibit the activity of JNK by S-nitrosylating the thiol group of JNK as endogenously produced NO has been shown to inhibit JNK activation in macrophages (Park *et al.*, 2000a). Another study showed that c-Jun, which is the main substrate of JNK and an important component of the AP-1 transcription factor, could be also nitrosylated which will inhibit its DNA binding activity (Klatt *et al.*, 1999). Furthermore, in HEK293 cells, NO was shown to directly interact with JNK1 causing suppression of this kinase (Park *et al.*, 2006). The authors illustrated that the mechanism of this suppression is cGMP-independent but that it is thiol-redox-sensitive, involving nitrosylation of Cys^{116} on JNK1. This contrasts with a study in mice which found that cGMP accounts for the anti-adhesive effects of NO (Ahluwalia *et al.*, 2004). Nitrosylation was also proposed to cause either steric constraint or unfavourable packing within the active site of JNK which ultimately disrupts the network of hydrogen bonds between JNK1 and the ATP molecule, preventing JNK using ATP for the phosphotransfer reaction (Hall *et al.*, 2000).

Others studies reviewed by Shen and Liu (2006) provided evidence that different RNS such as NO or $ONOO^-$ can induce the activity of JNK. It could be said that the actual effects of RNS on JNK depend on different factors like the actual RNS species, their

concentration, cell types and the presence of other stressing factors like ROS (Shen and Liu, 2006).

The effects of NO in modulating CAM expression are well established but the mechanism is still under debate. However, nitrosylation of some key proteins might play an important role in inhibiting CAM synthesis through JNK or NF-κB nitrosylation and/or modulation of CAM expression through inhibition of WPB exocytosis.

1.4.4. The relation between Hcy and NO

The bioavailability of NO is highly affected by Hcy levels (Stuhlinger *et al.*, 2001; Upchurch *et al.*, 1997) and Hcy-induced ROS consume NO and form the damaging peroxynitrite (Dayal *et al.*, 2002; Jacobsen, 2000; Lawrence de Koning *et al.*, 2003; Lim *et al.*, 2001; Loscalzo, 1996). However, the effects are on bioavailability as studies show that expression of eNOS is not suppressed by Hcy (Upchurch *et al.*, 1997; Zhang *et al.*, 2000). ADMA is an L-arginine analogue that acts as a competitive inhibitor of eNOS and therefore inhibits NO production (Boger, 2003). ADMA is metabolized to citrulline in an interaction which is catalyzed by an enzyme called dimethylarginine dimethylaminohydrolase (DDAH) (Boger, 2003). DDAH contains sulfhydryl groups in the Cys residues of its amino acid sequence which are critical for the enzyme's functionality and are thought to be a potential target for Hcy interference through forming disulfide bonds, inactivating DDAH (Stuhlinger *et al.*, 2001). Hcy was reported to inhibit DDAH in BAEC (Stuhlinger *et al.*, 2001). Furthermore, other studies in humans and animals reviewed by Dayal and Lentz (2005) reported that Hcy inhibits DDAH expression and activity leading to accumulation of ADMA and therefore inhibition of NO production (Boger, 2003; Boger *et al.*, 2001; Dayal and Lentz, 2005).

The relationship between NO and Hcy is complex however, as nitrosylation of Hcy by NO prevents it from generating ROS and from cyclizing to form thiolactone (Carmel and Jacobsen, 2001) a molecule which has deleterious effect 2-4 times greater than Hcy (Kerkeni *et al.*, 2006). Therefore, NO reduces the atherogenecity of Hcy.

$$R\text{-}SH + NO \longrightarrow R\text{-}SNO + H^{+} + e^{-}$$

S-nitrosothiols (SNO) have a broad spectrum of antiatherogenic properties (Stamler *et al.*, 1993). This group hypothesized that Hcy and NO exist in a balance within a healthy vascular system and the effects of Hcy may be tightly regulated by production of NO from endothelium, which converts it to S-nitrosohomocysteine. However, high levels of Hcy will result in theolactone formation leading to oxidative stress and ROS scavenging NO and leading to an imbalance between Hcy and NO levels. The end result could be uncontrolled Hcy production and decreased S-nitrosohomocysteine. Finally, Carmel and Jacobsen (2001) suggested that NO converts Hcy from "pathological mediator into physiological weapon" against atherosclerosis and they postulated that the ratio of NO to Hcy provides a better marker for atherogenic plasma than total Hcy in plasma (Carmel and Jacobsen, 2001). On the other hand, different studies reported that NO down-regulates MS in rat liver (Nicolaou *et al.*, 1997), platelets (Nicolaou *et al.*, 1994a) and rat brain synaptosomes (Nicolaou *et al.*, 1994b). MS was reported as a key enzyme in Hcy remethylation pathway (Lawrence de Koning *et al.*, 2003). Therefore, it has been suggested that this inhibition breaks the Hcy remethylation pathway thus leading to Hcy accumulation. Furthermore, this inhibition leads to a decrease in the SAM level and therefore affects metabolic pathways and metabolite levels that mediate signal transduction. Nicolaou's group demonstrated that these results might explain the cytotoxic effects of NO in CVD once present in excess and

also suggested a different kind of relationship between NO and Hcy where NO works as promoter for Hcy accumulation. However, none of these studies investigated the effects of accumulating Hcy on the studied cells.

These data could be merged together in a possible theory, under healthy conditions (Figure 11), NO which is produced moderately in EC regulates the MS activity thus maintaining balanced remethylation of Hcy. On the other hand Hcy might play a role as a negative regulator of NO production by controlling eNOS activity (via direct disulphide bond formation or interference with ADMA) in order to avoid NO overproduction thus avoiding MS inhibition and Hcy accumulation. Once NO is produced in excess (Figure 11), it will inhibit MS leading to accumulation of Hcy. Two possibilities arise here, NO might interact with the excess Hcy forming S-nitrosothiols and therefore overcoming the atherogenic effects of excess Hcy. If NO was not able to overcome overproduced Hcy, then this Hcy might induce ROS production which will interact with NO forming peroxynitrite which will be responsible for different atherogenic effects. The relationship between Hcy and NO is very complicated and still needs further investigation.

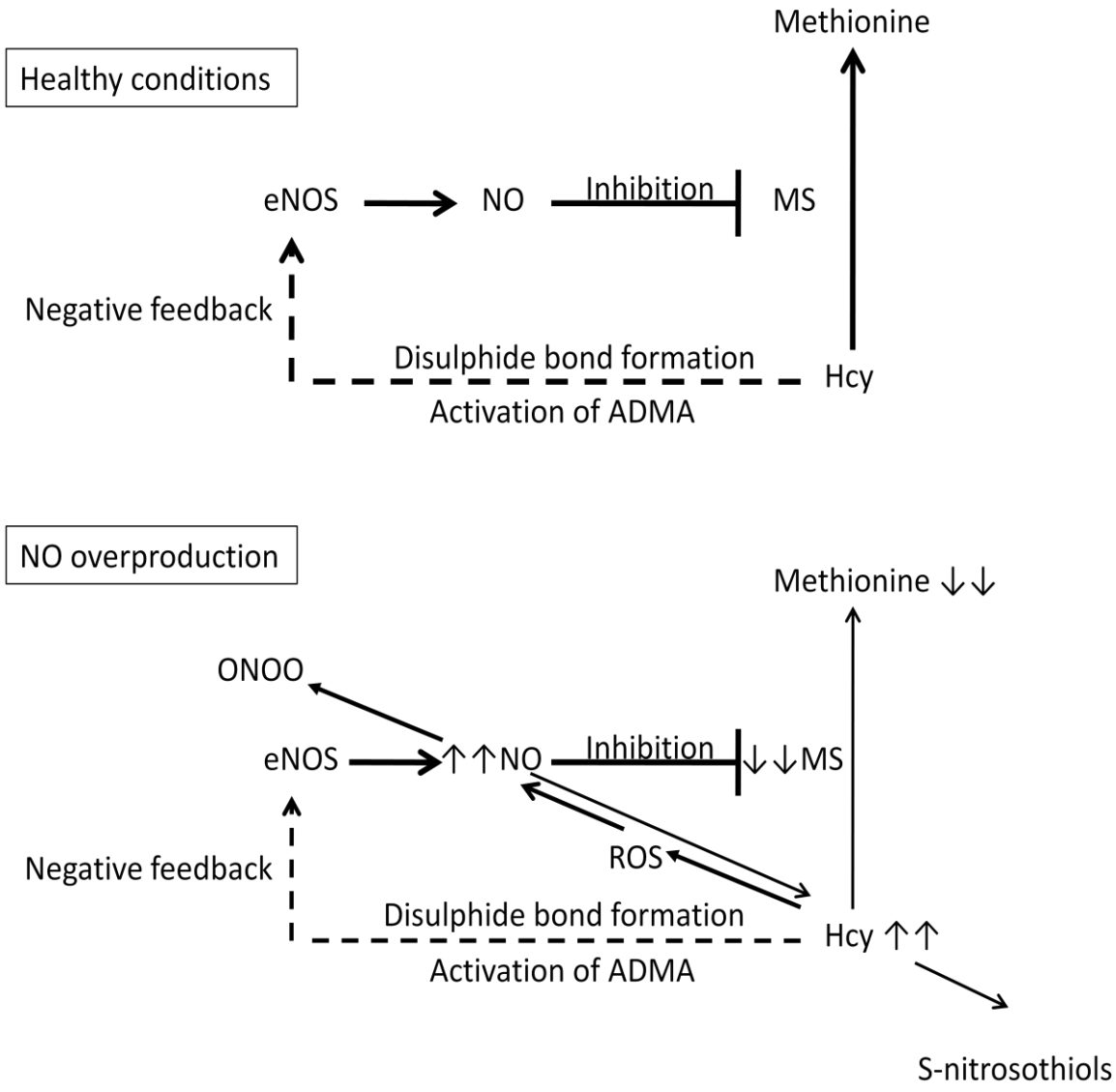


Figure 11: The relation between Hcy and NO under healthy and unhealthy conditions.

1.4.5. eNOS uncoupling

Under a number of pathological conditions, NOS enzymatic activity is disturbed and NOS starts producing $O_2^{\bullet-}$ at the expense of NO. This phenomenon is termed NOS uncoupling (Xia *et al.*, 1998). Xia's group showed that $O_2^{\bullet-}$ generation by eNOS is still a calcium/calmodulin-dependent process suggesting that $O_2^{\bullet-}$ synthesis requires electron transfer from the reductase domain to the oxygenase domain. This $O_2^{\bullet-}$ interacts with NO to form peroxynitrite decreasing vascular protection and eventually eNOS becomes an enzyme that generates only $O_2^{\bullet-}$. Both nNOS and iNOS were shown to produce $O_2^{\bullet-}$ in L-arginine depleted cells (Pou *et al.*, 1992; Xia and Zweier, 1997).

$O_2^{\bullet-}$ will interact with NO producing $ONOO^-$ which is a destructive molecule causing oxidation of nearly all organic molecules. Peroxynitrite ($^-O-O-N=O$) chemical reactivity is due to its easy protonation and the easy O−O bond cleavage in the transconfiguration of its acid form (Ullrich and Bachschmid, 2000).

$$^-OO-N=O \longrightarrow H-O-O-N=O \longrightarrow NO_2^{\bullet} + OH^{\bullet}$$

It was reviewed that $O_2^{\bullet-}$ generation by NOS can be due to the failure of the enzyme to form dimers and that the NOS monomers were able to generate $O_2^{\bullet-}$ although the enzyme has less enzymatic efficiency than the dimeric form (Forstermann and Munzel, 2006). NOS catalyse flavin-mediated electron transfer from the C-terminally bound NADPH to the haem on the N terminus. However, eNOS uncoupling happens when the electron flow within NOS is disturbed, then the ferrous-dioxygen complex dissociates and electrons flowing from reductase domain to oxygenase domain are diverted to molecular oxygen rather than to L-arginine (Forstermann and Munzel, 2006). Therefore, $O_2^{\bullet-}$ is

generated from the oxygenase domain instead of NO and thus dysfunctional eNOS produces ROS at the expense of NO (Xia *et al.*, 1998).

Maintenance and stabilization of NOS dimers are dependent on BH_4 (Channon, 2004) which is a critical cofactor for the efficient function of NOS. If BH_4 level is decreased for any reason like excess amount of ROS, eNOS starts producing $O_2^{\bullet -}$ instead of NO (Forstermann and Munzel, 2006; Landmesser *et al.*, 2003; Topal *et al.*, 2004; Zhang *et al.*, 2000). Generated oxidative stress will oxidize BH_4 to $BH_3^{\bullet}$ radicals within seconds and accelerates BH_4 degradation (Topal *et al.*, 2004) which enhances eNOS uncoupling. Folate stimulates the regeneration of endogenous BH_4 from dihydrobiopterin (BH_2) (Cines *et al.*, 1998) and that could explain the ability of folate to restore the lost NO-generating activity of the enzyme. Furthermore, $BH_3^{\bullet}$ can be reduced back to BH_4 by appropriate reducing agents like vitamin C which will lead to eNOS recoupling (Kuzkaya *et al.*, 2003). Hcy might decrease the NO release through oxidative stress in EC, which could lead to conversion of BH_4 to BH_2 and inhibition of NO-generating activity of eNOS (Zhang *et al.*, 2000). Furthermore, Topal *et al* (2004) found that Hcy (100µM/24h) decreased by 80% the intracellular amount of BH_4 in HUVEC leading to eNOS uncoupling. Furthermore, this group and others (Xia *et al.*, 1998) also found that Hcy-induced ROS generation was markedly blocked by NOS inhibitor L-NAME. Collectively, the previous data suggest eNOS uncoupling as a possible mechanism for Hcy-induced oxidative stress.

L-arginine depletion has also been shown to cause NOS uncoupling *in vitro.* However, the K_M of eNOS for L-arginine is 3µM *in vivo* and L-arginine plasma concentration is about 100µM with nearly 10-fold accumulation within cells so it has been argued that it hardly falls below 60µM. Furthermore, human EC can effectively recycle L-

citrulline to L-arginine and can obtain L-arginine directly from protein breakdown. On the other hand, EC expresses arginase that competes with eNOS for substrate, and if highly expressed "starves" eNOS (reviewed by Förstermann and Münzel (2006)). The same group reported enhanced arginase activity in HUVEC 24h after stimulation with inflammatory cytokines. Hcy was reported to decrease L-arginine intracellular uptake which was suggested also to induce eNOS uncoupling (Jin *et al.*, 2007).

In summary, under conditions of depleted L-arginine or BH_4, electrons derived from NADPH oxidation are transferred to oxygen molecules leading to $O_2^{\bullet-}$ formation (Xia *et al.*, 1998). This study showed that $O_2^{\bullet-}$ generation is a common feature of all NOS isoforms. nNOS and iNOS ability to generate $O_2^{\bullet-}$ is triggered by low levels of L-arginine or BH_4. However, Xia's group study found that eNOS ability to generate $O_2^{\bullet-}$ is not affected by the presence of L-arginine while BH_4 blocks $O_2^{\bullet-}$ formation in a dose dependent manner. Therefore, they concluded that "insufficient BH_4 availability will switch eNOS from NO to $O_2^{\bullet-}$ generation leading to NO decrease and ROS accumulation" (Xia *et al.*, 1998). As BH_4 plays a key role in maintaining eNOS dimerization, the conversion of eNOS dimer to monomer affects the interchange of NO/$O_2^{\bullet-}$ generation from this enzyme.

1.5. THE EFFECTS OF CHRONIC EXPOSURE TO DL-HCY ON ENDOTHELIAL CELL CYTOKINE PRODUCTION

1.5.1. Cytokines and their role in the inflammatory response

Cytokines are a category of signalling molecules that could be proteins, peptides or glycoproteins and includes more than 50 secreted factors involved in regulating different cellular functions in addition to intercellular communications and regulation of fundamental biological processes including body growth, lactation, adiposity, and hematopoiesis (Tedgui and Mallat, 2006). Inflammation is the tissue response to damage in an attempt to restore homeostasis and is highly controlled by different factors including cytokines. Cytokines play key roles in mediating inflammation and they participate in both acute and chronic inflammation. Cytokines like IL-1, IL-8 and TNF-α are involved in acute inflammation and are responsible for the primary response (Feghali and Wright, 1997). However, prolonged up-regulation of such cytokines will lead to the chronic inflammatory response (Muller, 2002). Cytokines are classified depending on their involvement in inflammatory response into two major groups, pro-inflammatory like TNF-α, INF-γ and IL-1 and anti-inflammatory like IL-4, IL-10 and IL-13 and there is a balance between pro- and anti-inflammatory cytokines which is crucial for lesion development (Kleemann *et al.*, 2008). Therefore, any factor that induces and maintains pro-inflammatory cytokines and thus induces imbalance toward the pro-inflammatory cytokines level will favour the development of atherosclerosis.

Chemokines are a subfamily of cytokines characterized by well conserved Cys residues (Harada *et al.*, 1996). They are small chemotactic cytokines such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) which both play a key role in recruiting the

inflammatory cells in the intima during acute inflammation, but also during the development of chronic inflammation such as atherosclerosis (Reape and Groot, 1999). Chemokines need to bind to their coupled receptors on the target cells to induce cellular changes and several studies in animal models, which are reviewed by Braunersreuther *et al* (2007) suggest that blocking chemokines:chemokine receptor interactions may serve as a suitable approach to treat atherosclerosis.

Different signalling pathways can be triggered in response to pro-inflammatory cytokines like JNK/AP-1, which has already been discussed in detail in this thesis (Chapter 3), and NF-κB. Both JNK and NF-κB signalling pathways have common downstream effects in regulating the expression of several genes encoding for other pro-inflammatory cytokines, chemokines and adhesion molecules (Bevilacqua, 1993; Read *et al.*, 1997; Zhou *et al.*, 2007) all of which play key roles in the inflammatory response and the initiation of atherosclerosis. Dysregulation of cytokine expression in vascular cells may contribute to the atherosclerotic process and some studies reviewed by Kleemann *et al* (2008) and Tedgui and Mallat (2006) have demonstrated that TNF-α, IL-8 (Rus *et al.*, 1996), IL-1, IL-2, IL-6 and INF-γ are present in atherosclerotic lesions.

1.5.2. IL-8 and its role in DL-Hcy induced EC activation

IL-8 is a member of C-X-C class of chemokines. It is produced by macrophages and other cell types such as EC which store IL-8 in WPB (Wolff *et al.*, 1998). IL-8 was found to be highly expressed in atherosclerotic lesions (Apostolopoulos *et al.*, 1996). It has been suggested to play a key role in recruiting leukocytes to vessel walls through mediating the firm adhesion of rolling neutrophils on EC (Gerszten *et al.*, 1999; Rot, 1992). This chemokine not only works as a chemoattractant, but also is essential for the conformational

changes of leukocyte integrins into the adhesive form and thus triggering firm adhesion (Gerszten *et al.*, 1999). Therefore, dysregulation of expression of this chemokine in vascular cells may contribute to the atherosclerotic process (Apostolopoulos *et al.*, 1996; Poddar *et al.*, 2001). IL-8 mechanism of action has been suggested to depend on its binding to heparin and heparan sulphate present on EC or in the basement membrane and which will enhance neutrophil chemotactic activity (Harada *et al.*, 1996).

TNF-α-mediated IL-8 gene transcription in EC has been shown to require cooperation between transcription factors like AP-1 and NF-κB (Natarajan *et al.*, 2001). The same group has showed that NO represses JNK-induced phosphorylation of c-jun and that leads to deactivation of the downstream transcription factor AP-1. Disruption of the JNK pathway leads to repression of the expression of IL-8. Furthermore, NO inhibits cytokine-induced IL-8 expression in EC through its effects on NF-κB activity (De Caterina *et al.*, 1995). Therefore, in addition to the other mechanisms (described in Chapter 5), NO can additionally reduce endothelial injury by modulating IL-8 expression.

Acute treatment with Hcy induced the production of IL-8 protein and mRNA in cultured EC (Poddar *et al.*, 2001). However, the effects of long treatment with Hcy on cultured EC have not been addressed. Hcy was also suggested to augment cytokine-mediated IL-8 expression in VSMC at both protein and mRNA levels (Desai *et al.*, 2001). Collectively, all of this data suggests a key role for IL-8 in mediating Hcy proatherosclerotic effects.

1.5.3. TNF-α and its role in DL-Hcy atherogenic effects

TNF-α is a pleiotropic cytokine with a potent effector pro-inflammatory function that is involved in systemic inflammation (Kleemann *et al.*, 2008). It functions through two distinct receptors, TNFR-1 and TNFR-2 causing activation of two major transcription factors, AP-1 and NF-κB which in turn activate transcription of several genes involved in chronic and acute inflammatory responses (Wajant *et al.*, 2003). TNF-α is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophages and neutrophils to a site of infection. It can regulate the selection between pro-apoptotic and anti-apoptotic signalling pathways, and the control of cell proliferation and inflammation (Baud and Karin, 2001).

TNF-α expression and/or signalling are implicated in a number of inflammatory diseases reviewed by Baud and Karin (2001) including Crohn's disease, rheumatoid arthritis and neuropathologies such as stroke. Furthermore, TNF-α has been associated with an elevated risk of recurrent myocardial infarction and cardiovascular death after a first myocardial infarction (Bennet *et al.*, 2006; Tedgui and Mallat, 2006). TNF-α is also higher in diabetics who have elevated CVD risk (Abdel Aziz *et al.*, 2001). Data about TNF-α involvement in the initiation and/or progress of atherosclerosis is still contradictory. *In vivo* studies reviewed by Galkina and Ley (2009) showed that knocking out TNF-α in *Apoe*$^{-/-}$ mice resulted in reductions in lesion formation (Ohta *et al.*, 2005). However, knocking out TNFR caused development of larger lesions compared to the control group (Schreyer *et al.*, 1996). Furthermore, Blocking TNF-α suppresses inflammation in ~70% of patients with rheumatoid arthritis (Sattar *et al.*, 2007) and is used widely in the treatment of chronic inflammatory and autoimmune disorders (Hurlimann *et al.*, 2002; Ohta *et al.*, 2005).

Despite these encouraging results for TNF-α blockers, one clinical study of only one case suggested that TNF-α blockers can induce plaque rupture (Settergren and Tornvall, 2004). It could be said that the role of TNF-α in inducing EC dysfunction leading to atherosclerosis initiation was studied intensively (Zhang *et al.*, 2009). A very few studies have reported positive correlation between TNF-α and Hcy levels in patient with high risk of CVD (Abdel Aziz *et al.*, 2001; Akalin *et al.*, 2008). However, the actual role of TNF-α in initiating and mediating Hcy-mediated inflammatory responses is still unclear.

Macrophages and monocytes represent the primary source for TNF-α in the site of injury (Kleemann *et al.*, 2008) and TNF-α effects on the EC have been studied intensively and shown to induce EC activation/dysfunction (Eccles *et al.*, 2008; Xia *et al.*, 2006; Zhou *et al.*, 2007). However, most studies used exogenous TNF-α to induce EC activation/dysfunction. Therefore, it is still unclear whether leukocytes are the main source of TNF-α in atherosclerotic lesion *in vivo* and whether EC can produce TNF-α and contribute to its total production in atherosclerotic lesion. Therefore, we will investigate whether EC express any basal production of TNF-α and whether Hcy can induce such production.

1.6. AIMS

HUVEC were used to study the functional chronic effects of Hcy as a model for atherosclerotic plaque formation and the mechanisms resulting in these effects. Therefore the aims were as follows:

- Investigation the effects of chronic treatment with Hcy on HUVEC viability and proliferation rate.
- Assess the expression of ICAM-1, E-selectin and P-selectin on EC surface in response to Hcy chronic treatment under static conditions.
- Use an *in vitro* flow model to study the functional effects of Hcy chronic treatment on EC:neutrophil interactions under flow conditions.
- Study the effects of chronic Hcy exposure on the activation of JNK and its downstream substrate c-Jun and the role of activated JNK in mediating P-selectin, E-selectin and ICAM-1 expression in response to chronic Hcy exposure under static and flow conditions by using a specific JNK inhibitor.
- Assessment of whether Hcy induces ROS generation in HUVEC and therefore induces oxidative stress and the involvement of Hcy-induced oxidative stress in JNK pathway activation.
- Study of the involvement of Hcy-induced oxidative stress in HUVEC:neutrophil interactions.
- Investigation of the benefit of antioxidants in preventing all of the previous effects.
- Investigation of the effects of NO donors on DL-Hcy-induced HUVEC:neutrophil interactions and also investigate the mechanism of action of these donors.

- Study the effects of inhibiting eNOS by L-NAME on DL-Hcy-induced superoxide anion generation in HUVEC and on HUVEC:neutrophil interactions.
- To quantify the effects of chronic treatment with Hcy on IL-8 and TNF-α protein production and on IL-8 and TNF-α mRNA expression in HUVEC.

CHAPTER 2

2. THE ENDOTHELIUM AND HOMOCYSTEINE

Atherosclerosis is currently well defined as a chronic inflammatory disease of the wall of large arteries (Galkina and Ley, 2009). Due to its role in maintaining vascular homeostasis, anything that causes dysfunction of EC could be considered among the primary causes of atherosclerosis (Davignon and Ganz, 2004). Some researchers (Deanfield *et al.*, 2007) tend to refer to endothelial dysfunction as endothelial activation where quiescence (a feature mainly mediated by NO) is lost and cells play a role in disease development due to alteration in redox signaling. Endothelial dysfunction or activation *in vivo* can be assessed by different markers such as increased plasma levels of EC-generated molecules like von Willebrand factor (vWF), numbers of circulating EC and endothelial progenitor cells as well as vascular activity to an appropriate stimulus (Brevetti *et al.*, 2008). EC activation *in vitro* can be assessed by up-regulation of several cell adhesion molecules as well as increases in the different types of interactions between leukocytes and EC.

One independent risk factor for atherosclerosis present in up to 7% of the population is hyperhomocysteinemia. Since its identification as a risk factor over 30 years ago (McCully, 1969), many studies have identified the involvement of homocysteine (Hcy) in atherosclerotic plaque formation (Nygard *et al.*, 1998; Selhub *et al.*, 1995; Tsai *et al.*, 1994). There is abundant evidence for homocysteine mediated damage of endothelial cells *in vitro* (Chang *et al.*, 2008; Dalal *et al.*, 2003; Koga *et al.*, 2002; Outinen *et al.*, 1999) *and in vivo* (Hofmann *et al.*, 2001) which is likely to be a key step in accelerated atherogenesis

since EC represent the primary site for the initiation of atherosclerosis. Therefore, in this chapter, Hcy-induced activation of EC in culture was investigated by characterizing EC which were chronically treated with Hcy. That involved assessment of Hcy effects on EC morphology, proliferation, expression of CAM and their interactions with neutrophils under flow conditions.

2.1. MATERIALS AND METHODS

2.1.1. MATERIALS

2.1.1.1. Cell culture

All tissue culture reagents used were of tissue culture grade. Endothelial cell growth medium (C-22020) was purchased from Promocell (Sickingenstrasse, Germany). Human serum was obtained from Lonza (14-402F, Biowhittaker Lonza, Walkersville, MD, USA) or from the Blood Transfusion Services, Sheffield. Commercial serum was used directly while serum from the Blood Transfusion Services was centrifuged after collection at 3000*g* for 15min at 4°C and sterile filtered using 0.22μM bottletop filters from Corning Costar (High Wycombe, Bucks, UK). The serum was stored in 50ml aliquots at -20°C. Dulbecco's Phosphate Buffered Saline (DPBS, BE17-513F and BE17-512F) was obtained from Lonza, (Verviers, Belgium). Penicillin/streptomycin (15140-114), L-glutamine (25030-024), Amphotericin B (fungizone, 15290-026), trypsin/ethylenediaminetetraacetic acid (EDTA) (45300-019), Hanks Balanced Salt Solution (14025-050) and RPMI-1640 (52400) were obtained from Gibco (Paisley, Scotland, UK). Lymphoprep (NYC-1114545) was purchased from Axis Shield. Heparin sodium (PL 0043/0038R, PA 46/3612) was supplied by Leo Laboratories limited, Bucks, UK. All tissue culture plastics used: 25cm^2 and 75cm^2 flasks, 35mm dishes, 96-well plates and 6-well plates were either from Corning Incorporation (Corning, NY 14831, USA) or Becton-Dickinson (Falcon), (Leeds, England UK). Disposable sterile pipettes of volume 5, 10 and 25ml were from Costar Corporation, Cambridge or from LIP (Equipment & Services) Ltd, Shipley, England. Disposable syringes were purchased from National Health Supplies (Leeds, UK).

Medium 199 (M-2154), collagenase type IA (C/N C-2674), dextran (M.W. 513,000, D-5251), dextrose (G-7021), histamine dihydrochloride (H-7250), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES, H-0891), tris base (T-6066), bovine serum albumin (BSA, A-4503), gelatine (G1393), trypan blue (T6146), dimethylsulphoxide (DMSO, D-8779), DL-homocysteine (H-4628), L-cysteine (C-7352), SP600125 (S-5567), recombinant human tumour necrosis factor-alpha (TNF-α, T-0157), normal donkey serum (D9663), paraformaldehyde (P6148) and MTT (M2003) were all purchased from Sigma-Aldrich Chemical Company (Poole Dorset, UK).

2.1.1.2. Antibodies

ELISA

Mouse monoclonal antibodies against human intercellular cell adhesion molecule 1 (ICAM-1) (CD54, C/N BBA3, clone #BBIG-I1 (11C81)), E-selectin (CD62E, C/N BBA16, clone #BBIG-E4 (5DII)) and P-selectin (CD62P, C/N BBA30, clone #9E1) and negative control antibody mouse IgG_1 (C/N MAB002) were obtained from R&D Systems, Oxon, UK. Polyclonal rabbit anti mouse immunoglobulins-HRP (P.0260) was obtained from Dakocytomation Glostrup, Denmark.

Immunofluorescence

Fluorescein-conjugated mouse monoclonal antibodies against human intercellular cell adhesion molecule 1 (ICAM-1) (CD54, C/N BBA20, clone #BBIG-I1), E-selectin (CD62E, C/N BBA21, clone #BBIG-E5 (10C10)) and P-selectin (CD62P, C/N BBA34, clone #9E1) were obtained from R&D Systems, Minneapolis, MN, USA. Fluorescein-conjugated sheep polyclonal antibodies against human Von Willebrand Factor (ab8822) were obtained from abCAM, Cambridge, UK. Fluorescein-conjugated mouse monoclonal

antibodies against human CD31 (PECAM-1) (F8402) were obtained from Sigma-Aldrich Chemical Company (Poole Dorset, UK).

2.1.2. METHODS

2.1.2.1. Cell culture

2.1.2.1.1 Collection of umbilical cords

Umbilical cords were collected in the Maternity unit in the Bradford Royal Infirmary by nurses and midwifery staff from healthy donors with consent of the local ethical board (LERC NO: 03/12/433) (ethical approval letter is attached: Appendix 4) in RPMI 199 medium containing penicillin/streptomycin (100U/100mg/ml), fungizone (2.5µg/ml) and heparin (10U/ml) and stored at 4°C until isolation of EC. Samples from donors with inflammatory diseases, diabetes, smokers or those using anti-inflammatory medications were excluded from the experiments (data sheet for each donor is displayed in Appendix 5).

2.1.2.1.2. Isolation of human umbilical vein endothelial cells (HUVEC)

EC were isolated using an enzymatic technique, based on a procedure developed in the University of Bradford (Lim, 1995). The veins were first washed to remove any blood clots and cell debris by flushing through phosphate buffered saline (PBS) containing penicillin/streptomycin (100U/100mg/ml) and fungizone (2.5µg/ml) using a disposable syringe. Following flushing, the vein was clamped at one end and filled with 0.1% v/v collagenase in serum free medium 199. The vein was then clamped at the top and incubated for 15min in an atmosphere of 95% air/CO_2 at 37°C. Collagenase solution was collected after incubation and the vein flushed through twice with PBS containing antibiotics and once with air to remove any residual EC detached by the collagenase. The resulting

suspension was centrifuged at 560*g* for 5min at 20°C. The supernatant was discarded and the pellet resuspended in 5ml of complete medium (M199-M2154 containing 20% v/v human serum, penicillin/streptomycin (100U/100mg/ml), fungizone (2.5µg/ml) and L-glutamine (2mM)). The cell suspension was plated into a T25 culture flask and into a T75 culture flask if the cord was very long. All cell culture plastics used for the culture of HUVEC were coated in 10% v/v bovine gelatin in PBS prior to use for at least 30min and rinsed with PBS prior to addition of cells.

2.1.2.1.3. Culture of HUVEC

After isolation, cells were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C. Medium was changed 24h later to remove any dead cells, cell debris and collagenase traces and cells were washed twice with warm PBS. Subsequently, medium was changed every 48h. HUVEC were used up to passage 4 as they have been characterized within the lab (Cooper, 2000) to respond to stimuli in a manner consistent with newly isolated endothelium until passage 4. In addition, the endothelial phenotype was confirmed using phase-contrast microscopy and immunostaining for vWF and CD 31 (PECAM-1: platelet endothelial cell adhesion molecule-1) in addition to the distinct cobblestone like arrangement at confluence.

2.1.2.1.4. Immunocharacterisation of EC

Chamber slides (Nunc, Roskilde Denmark) were seeded with a concentration of 2 x 10^5 cells per ml (500µl/chamber). Confluent monolayers of HUVEC were fixed using 2% w/v paraformaldehyde (in PBS) for 10min at 4°C and then washed thoroughly with PBS. The cells were blocked with blocking buffer (5% v/v donkey serum and 1% w/v BSA in PBS, 90min) and then they were incubated with 200µl sheep polyclonal anti human vWF

antibodies (1:20) or mouse monoclonal anti human CD31 antibodies (1:20) for 2h at 4°C. Cells from same donors were incubated with FITC-tagged rabbit anti-mouse immunoglobulin antibody and considered as negative control. Vectashield with 4',5-Diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, CA, USA) was used to counter stain nuclei. Cells were viewed on an Eclipse TE2000 inverted research microscope (Nikon, Tokyo, Japan) and photographed with a cooled Hamamatsu digital camera (Hamamatsu, Japan) using 20x objective. Photos of randomly chosen fields were taken in each experiment using the FITC- filter and DAPI- filter for antibodies-treated and non-treated cells and compared to check the basal.

2.1.2.1.5. Expansion of cultures

When cells were confluent, cells were passaged at a ratio of 1:3. Culture media was discarded and cells were rinsed twice with PBS and then treated with Trypsin/EDTA (0.05%/0.02%) to detach the cells from the base of the flask. Cells were observed under the microscope and the trypsin was removed as the cells started rounding up i.e. losing cell-cell contacts. The flask was then incubated at 37°C for approximately 1min, and then the flask was tapped against the palm to detach the cells. Detached cells were resuspended in an appropriate volume of complete medium M199, seeded into the appropriate container and returned to the incubator. 24h later, cells were washed with warm PBS and the medium was changed. Consequently, medium was changed every 48h until confluence was reached.

2.1.2.1.6. Cryopreservation of cell stocks

Cells were cryopreservred between passage 2 and 4. After trypsinizing the cells as described above in section 2.1.2.1.5, cells were resuspended in complete M199 media and centrifuged at 560*g* for 5min at room temperature to repack the cells. The cell pellet was

resuspended in 4°C foetal calf serum containing 10% v/v DMSO. Cells were frozen at a density of $1x10^6$ cells/ml. cells were placed in cryovial and placed immediately on ice. The cryovials were wrapped in layers of tissue paper and placed in -80°C for 24h before they were transferred to liquid nitrogen Dewar flasks and stored until required.

2.1.2.1.7. Resuscitation of cells

In order to maintain cell viability, resuscitation of frozen cells was carried out as quickly as possible. The cryovial of frozen cells was removed from liquid nitrogen and placed in a water bath at 37°C. Once thawed, the cells were transferred to a T75 cell culture flask and resuspended in appropriate volume of complete M199 media. 24h later, cells were washed with warm PBS and the medium was changed to remove any traces of DMSO and any dead cells. Consequently, medium was changed every 48h.

2.1.2.1.8. Determination of cell concentration and cell viability

When required, the concentration of the cells was determined by counting in a haemocytometer under light microscopy. Cells were trypsinized off the flask, as described in section 2.1.2.1.3 and resuspended in 10ml complete M199 medium. 10µl cell suspension was loaded onto each chamber of a standard haemocytometer and cells were allowed to settle randomly over the grid. The total amount of the cells in an area of 0.4 mm^2 (within four corners from the grid) was counted under the light microscope. The cell concentration was calculated according to the formula:

$$\text{Cell concentration (cells/ml)} = \frac{TC}{4} \times \text{Dilution factor} \times 10^4$$

Trypan blue, a vital dye, was used to detect cell viability. Viable cells exclude the trypan blue whilst this dye enters to the non-viable cells and which are easily identified during counting in the haemocytometer. Dyed cells and non-dyed cells were counted and the percentage viability was calculated as follows:

Unstained cells (viable)/total cells (viable and non-viable) X 100.

2.1.2.2. Chronic treatment of EC with DL-Hcy

HUVEC were cultured for a period of 5-9 days in complete M199 medium containing 1mM DL-Hcy allowing cells to undergo at least one passage in the presence of DL-Hcy. Following the period of exposure to DL-Hcy or Cys, HUVEC were subjected to different kinds of treatment depending on the experiment which will be conducted as described in the following sections.

2.1.2.3. Negative controls

Cells were treated with Cys in the same way as they were treated with Hcy using similar concentrations (1mM for 5-9 days). Cys served as an osmotic control to ensure that the observed effects were due to Hcy and not due to osmotic stress which might have activated EC. Cys has a similar structure to Hcy with a similar free thiol group and therefore, it has also been used as a negative control to check that the observed effects with Hcy were specific to Hcy and not nonspecific effects from a free thiol group. Therefore, two negative controls were used in all the experiments: non-treated cells, which were grown in complete M199 media with no treatment and Cys-treated cells. These negative controls were used in the experiments in this chapter and all the following chapters. Due to

the limited availability of HUVEC, a smaller number of experiments using the negative control Cys were carried compared to those using Hcy or non-treated controls.

2.1.2.4. Cell proliferation rate (MTT assay)

To determine the HUVEC growth rate, an MTT assay was conducted. A solution of 3-[4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is colourless or yellowish. However, mitochondrial dehydrogenase of viable cells cleaves the tetrazolium ring of MTT, yielding purple MTT formazan crystals. These crystals can be dissolved in DMSO and the resulting colour read spectrophotometrically giving absorbance proportional to cell number. HUVEC were seeded in triplicate into gelatin-coated 96 well plates ($1x10^4$ or $2x10^4$ cells per well). Complete M199 was added to three wells of the plate to act as a cell blank control. Plates were incubated at 37°C and 5% CO_2 in a humidified atmosphere for 96h. Culture media was changed once after 48h. Following incubation, MTT solution (5mg/ml) was added aseptically in an amount equal to 10% of the culture volume and plates were incubated for 3-4h at 37°C and 5% CO_2 in a humidified atmosphere. Subsequently, media was discarded and the crystals formed were dissolved in 150µl DMSO. Within 1h absorbance was measured using Microplate reader MRX II (Dynex technologies, Chantilly, USA) at a wavelength of 570nm and blank readings were subtracted from those of samples. This technique was also carried out every 24h for 4 days to measure the daily proliferation rate of non-treated and treated HUVEC.

2.1.2.5. Assessment of HUVEC and their CAM expression by Enzyme-linked immunosorbent assay (ELISA)

96 well plates were seeded with a concentration of $2x10^5$ DL-Hcy-treated or control cells per ml ($2x10^4$ cell/well). Plates were incubated at 37°C and 5% CO_2 in a humidified

atmosphere overnight. Cells were fixed with 100µl of 2% w/v paraformaldehyde (in PBS) and then incubated with 1% w/v BSA in PBS (100µl per well) for 60min at room temperature to block non-specific binding. The mouse anti-human primary antibodies to ICAM-1, E-selectin and P-selectin were optimally diluted (2µg/ml) with 0.125% w/v BSA in PBS. The blocking buffer was removed and replaced with diluted antibodies. Plates were incubated overnight at 4°C. Excess antibody was removed by multiple washes (at least 3x) with the wash buffer, 0.05% v/v Tween 20 in PBS. The plate was inverted and blotted gently on paper tissue to remove as much wash buffer as possible before proceeding to the next step. The secondary antibody, rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (RAM-HRP), was diluted 1:1000 with the diluent buffer (0.125 % w/v BSA in PBS) and 100µl/well was added to all wells. Plates were incubated for 60min at RT, and then the washing steps repeated to remove any excess antibody. Substrate solution consisting of 50ml of 50mM phosphate-citrate buffer (pH 5.0), with o-phenylenediamine (OPD) dihydrochloride (3.7mM) and 20µl of 30% hydrogen peroxide was prepared fresh. 100µl/well of substrate was added and the soluble coloured end-product allowed to develop for 30min in the dark at RT. 50µl/well of 1M sulphuric acid was the last addition to stop the reaction and the absorbance was read at 490nm using a multiwell ELISA plate reader. Blank wells were used in this experiment and treated in the same way the sample wells were treated except that they did not have cells in them. All samples were tested in triplicate and the mean absorbance of the blank wells was then subtracted from the mean absorbance of each set of sample wells incubated with the different antibodies to exclude any nonspecific measurement.

2.1.2.6. Assessment of HUVEC and their CAM expression by immunofluorescence

Chamber slides (Nunc, Roskilde Denmark) were seeded with a concentration of 2 x 10^5 cells per ml (500µl/chamber). Confluent monolayers of HUVEC with treatments described previously (section 2.1.2.2) were fixed using ice cold 100% methanol for 10min at -20°C and then washed thoroughly with PBS. The cells were blocked with donkey serum (10% in PBS, 90min) and then they were incubated with 100µl mouse monoclonal anti-human E-Selectin/CD62E FITC (50µg/ml), anti-human P-Selectin/CD62P FITC (50µg/ml) or anti-human ICAM-1/CD54 FITC (75µg/ml) (R&D systems, Minneapolis, MN, USA) overnight at 4°C. Vectashield with 4',5-Diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, CA, USA) was used to counter stain nuclei. Cells were viewed on an Eclipse TE2000 inverted research microscope (Nikon, Tokyo, Japan) and photographed with a cooled Hamamatsu digital camera (Hamamatsu, Japan) using 20x objective.

Photos of four randomly chosen fields were taken in each experiment using the FITC- filter and DAPI- filter and the results were quantified using ImageJ software after adjusting the threshold. As DAPI counterstains nuclei, these images were used to make sure that roughly equal numbers of cells are present in each chosen field and hence any difference between FITC filter-captured photos were due to different intensity of FITC rather than different densities of cells.

2.1.2.7. Assessment of EC:neutrophil interactions under flow conditions

A flow chamber was used to subject cultured HUVEC treated and non-treated monlayers to laminar flow shear stress and mimic blood flow through a human blood vessel. To study the effects of DL-Hcy on the expression of the individual adhesion

molecules under flow conditions, HUVEC were cultured as indicated in section 2.1.2.2. Subsequently, HUVEC were subjected to flowing neutrophils.

2.1.2.8. Isolation of neutrophils

Neutrophils were isolated from whole blood obtained from consented healthy donors according to the regulations of the University of Bradford.

Blood was collected into sodium citrate (0.1M, pH: 6.5) 1:10. The blood was diluted with an equal volume of HEPES HBSS (Hanks Balanced Salt Solution, Invitrogen: 14025-050) (0.08M dextran and 0.025M HEPES in HBSS). Tubes containing the blood and HEPES HBSS solution were left to stand at room temperature for 45min. The top layer was taken carefully and added to universal tubes containing an equal amount of lymphoprep (Axis Shield: NYC-1114545). These tubes were centrifuged at 400*g* for 30min at 4°C then kept on ice. The supernatant was discarded carefully and the remaining RBC were lysed by the addition of 1ml ice-cold water per each neutrophil pellet and agitation for 1min. All pellets, including the sterile water were combined in one tube and mixed with DPBS (BioWhittaker: BE17-513F and BE17-512F) containing 0.5% v/v human serum. The suspension was re-centrifuged at 400*g* for 15min at 4°C. Then the pellet was resuspended in 10ml ice-cold DPBS/0.5% human serum and the neutrophil concentration was adjusted to 1×10^6 cells/ml. Neutrophil viability was routinely assessed using Trypan blue dye exclusion.

2.1.2.9. Flow chamber assay

Sterile 35mm cell culture dishes (BD Falcon, cat No. 351008) were coated with 10% v/v gelatine solution (in PBS) for at least 30min and rinsed with PBS prior to addition

of cells. 2ml EC suspension ($2x10^5$cell/ml) were seeded onto the dishes. Dishes were incubated overnight at 37°C in an atmosphere of 95% air and 5% CO_2 in order for the cells to form confluent monolayers. The 35mm dish was fitted with a gasket where removal of a 5.0 x 50.0mm rectangular section from gasket provided a flow channel, with a height of 0.25mm (the gasket thickness). The chamber has a vacuum outlet, and two ports (appendix 1). The neutrophil solution was fed through the first port while the other port was connected to syringe pump (Harvard apparatus, south Natick, MA) which pulled the neutrophil solution at a rate of 0.5ml/min which is equivalent to a shear stress equal to 1.1dyne/cm^2. The vacuum helped in holding the chamber to the 35mm cell culture dish on which the EC had been cultured.

The shear stress applied in the flow an experiment reflected a shear rate in venules in vivo and it was calculated using this formula (Lawrence *et al.*, 1987):

$$\tau = \frac{6Q\mu}{wh^2}$$

When, τ = shear stress (dyne/cm^2); Q = volumetric flow rate (ml/sec); μ = viscosity of the liquid (poise); w = width of the flow field (cm); h = height of the flow field (cm).

Medium covering confluent cells was removed and the chamber containing the gasket was put on the dish and held in place by vacuum. Existing air bubbles were carefully removed from the system, via a bubble port. The flow chamber was placed onto the stage of an inverted microscope (appendix 1). EC were visualized at 10x and 20x magnification using phase contrast microscopy. The clear dishes allowed direct live time microscopic observation of the EC in the dish under exposure to flow. The entire period of perfusion

was recorded via a charge coupled device camera and video recorder equipped with a time-date generator and millisecond clock and a black and white television monitor (appendix 1 photo B), then the film was converted into digital image in order to be quantified on the computer. Tethering, rolling, adherent and transmigrated cell were counted.

2.1.2.10. Classification of EC:neutrophil interactions:

2.1.2.10.1. Tethering

Neutrophil tethering on EC was detected within the first 6min of the flow using the x10 objective. Two kinds of tethering have been distinguished, primary and secondary tethering. Primary tethering interactions are weak reversible interactions which occur quickly between neutrophils and EC. Secondary tethering interactions are similar to the primary ones however, they occur between adherent and flowing neutrophils. Tethering interactions are characterised as such when the duration of the interaction is <1s.

2.1.2.10.2. Rolling

After the first 6min, neutrophils rolling on EC were detected for a further 8min of flow using the x20 objective. Rolling neutrophils were classified when they remained in contact with the EC for longer than the tethering time (>1s) and could be easily observed rolling on the EC monolayer. Rolling neutrophils have a greatly decreased velocity when compared to other flowing neutrophils and were easily distinguished from both tethering and fixed neutrophils.

2.1.2.10.3. Fixed

Neutrophils were considered as fixed when they made firm contact with EC and stayed there for the whole period of flow (14min). Fixed neutrophils were counted at the

start of the second phase of flowing (after the initial 6min) and the end of the whole flow process (14min) using x20 objective. Fixed neutrophils were distinguishable from other neutrophils as they exhibited no movement for the whole 14min duration of the experiment.

2.1.2.10.4. Transmigration

During this stage, neutrophils underwent morphological changes and they could be seen flattening and spreading. Their shape changed from bright field to a duller shade and had a characteristic "halo" appearance as they transmigrated through the EC monolayer. Transmigrated neutrophils were counted at the end of the 14min flow period.

2.1.2.11. Counting of EC:neutrophil interactions

All of the above described interactions were counted manually. A grid on transparent paper was designed to divide the computer screen into 9 portions. Therefore, counting was done within all areas of the screen in one time. Therefore, each grid was screened frame by frame for a given time point and for a one type of interaction only. This was repeated for the same time point and interaction type in all of the 9 grids on the screen. The same quantifying method was repeated for all of the remnant interactions. The results were presented graphically using the mean values of each type of interaction, +/- SD, counted in each flow experiment. A complete flow experiment usually contained between 4-12 35mm dishes depending on the types of stimulation.

The counting method was developed by Eccles (2006), where for each period of 6min during which both primary and secondary tethering were counted, just 10s at the end of each minute was counted and the figure multiplied by 6 to give the result for the whole minute. The same procedure was applied in counting rolling and a 10s of each minute of

the 8min was counted. Again the figure was multiplied by 6 to give a figure of the whole minute. The numbers of fixing and transmigration interaction were counted by the end of each minute.

2.1.2.12. Statistical analysis

Excel and SPSS software packages were used for the statistical analysis of the data in all chapters. We tested normal distribution using Kolmogorov–Smirnov test and values were assumed to follow normal distribution if p value was more than 0.05. For normally distributed values, all data analysis was carried out using paired t-test unless specified. The differences were considered significant if p values were < 0.05. The same statistical analysis was carried on in this chapter and all the following chapters unless specified.

2.2. RESULTS

2.2.1. Isolation and culture of primary HUVEC

The length of the cord sections was on average 15-30cm with a diameter of nearly 3-6mm. HUVEC were isolated as described in section 2.1.2.1.2. Under phase contrast microscopy, newly isolated HUVEC looked like small floating dots, they adhered within 12h to the gelatin coating the flask and they had a small irregular cigar shape (Figure 12A). These cells proliferated and became confluent within 24-48h in T25 flasks and 2-3days in T75 flasks (Figure 12B). The growth rate was variable between cells from different donors. Upon confluence, cells were passaged at ratio of 1:3 up to passage number 4. HUVEC which were not able to adapt to culture conditions were discarded. Total contact inhibition was not observed. However, cells proliferated very slowly when they reached confluence and the number of floating dead cells was higher in confluent flasks then non-confluent ones.

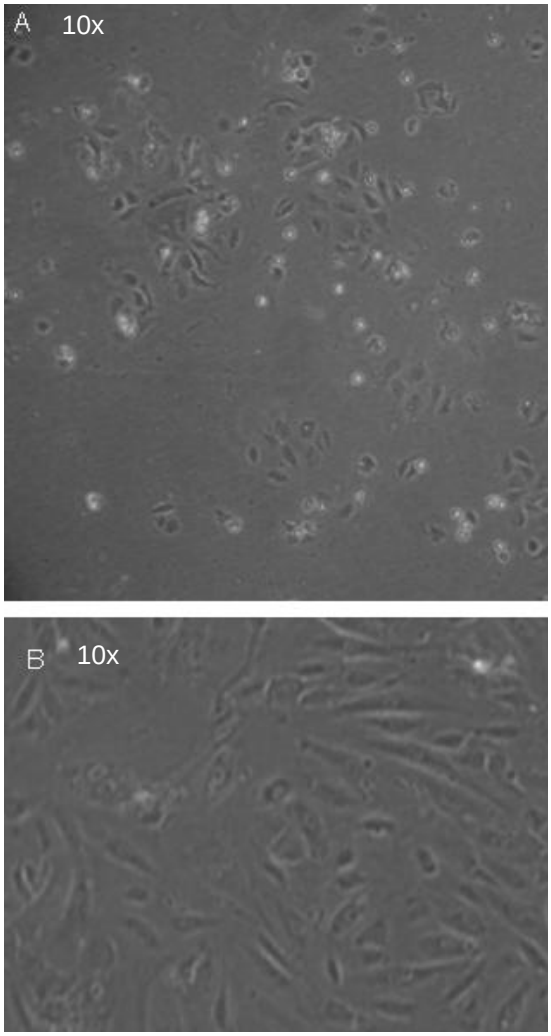


Figure 12: HUVEC in culture, A: primary HUVEC 24h after harvesting. B: confluent monolayer of HUVEC. (Original magnification is 100x).

2.2.2. Characterization of isolated EC

The endothelial phenotype was confirmed using phase-contrast microscopy and immunostaining for vWF and CD31 (PECAM-1) in addition to the distinct cobblestone like arrangement of the cells at confluence. FITC-tagged antibodies against vWF and CD31 were used to visualise their expression on the HUVEC surface. Both molecules gave high fluorescence in HUVEC indicating a basal expression of these molecules (Figure 13) while cells which have been treated with FITC-tagged rabbit anti-mouse immunoglobulin antibody showed no fluorescence (data not shown). Cells which did not show cobblestone like arrangement or did not express vWF and CD31 on their surface were excluded and not used in the experiments.

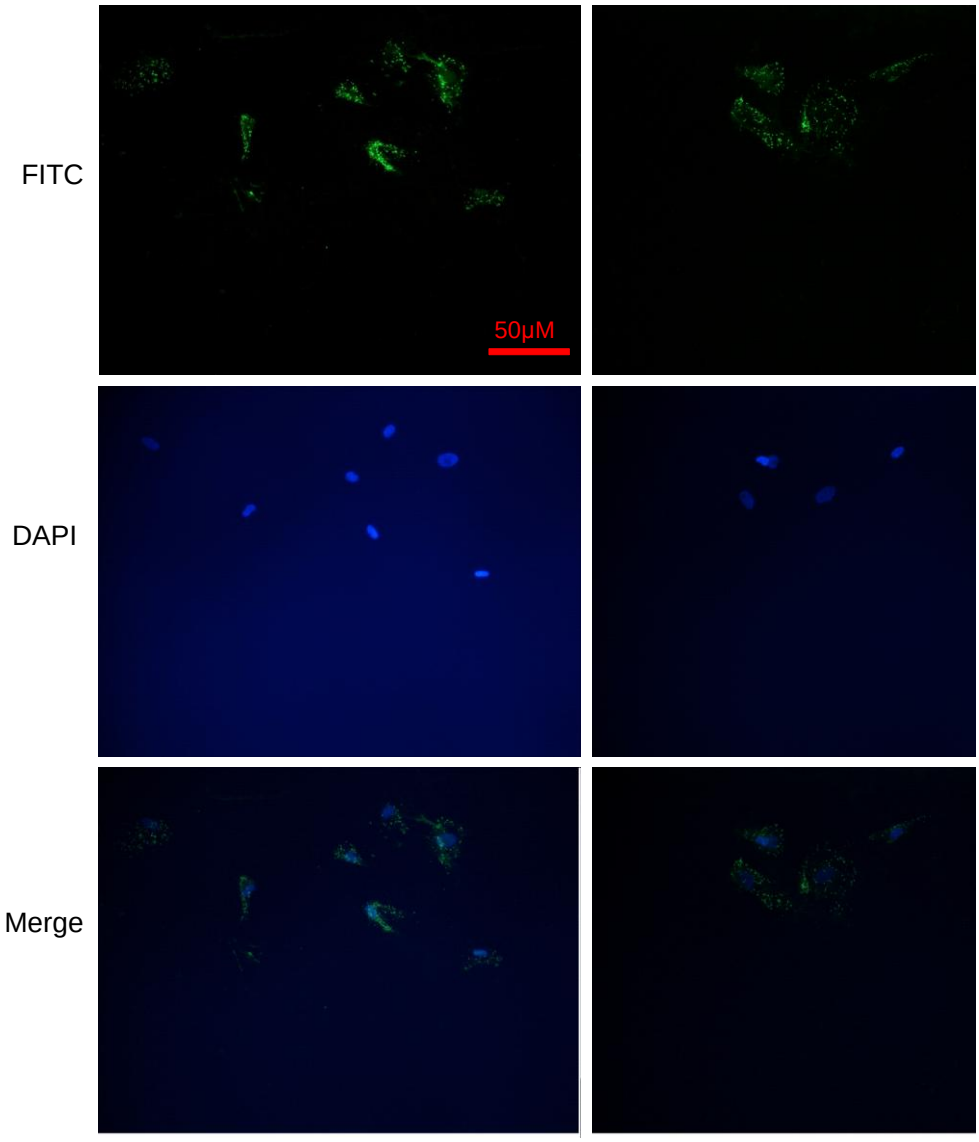


Figure 13: Representative images of the expression of vWF and CD31 on HUVEC surface. HUVEC were grown in complete M199 medium and then were fixed and immunostained with FITC-tagged antibodies against CD31 or vWF. The upper row is immunofluorescence microscopy detection of FITC, the middle raw is immunofluorescence microscopy detection of DAPI and the bottom one is merge of the two. (Original magnification 200x).

2.2.3. Characterization of chronic DL-Hcy effects on HUVEC

2.2.3.1. Morphological changes in HUVEC cultured in DL-Hcy

DL-Hcy appeared to affect the growth rate of the HUVEC when compared with non-treated HUVEC from the same donor. The effects seem to have an inverse relationship. There were also alterations in the phenotypic appearance of the cells. The difference was very obvious between HUVEC which had been grown in normal complete M199 media (Figure 14A) and those which had been grown in complete M199 media containing DL-Hcy at either 1mM or 5mM (Figure 14B, C respectively). The DL-Hcy-treated cells were elongated in shape and showed slower proliferation. In contrast, Cys did not seem to have negative effects on either growth rate or phenotypic appearance of the HUVEC when added to the media compared with non-treated HUVEC from the same donor (data not shown). Furthermore, individual differences could be noticed between cells from different donors in response to exposure to DL-Hcy. Photos in Figure 14B represent cells typically used in experiments to produce results in this thesis.

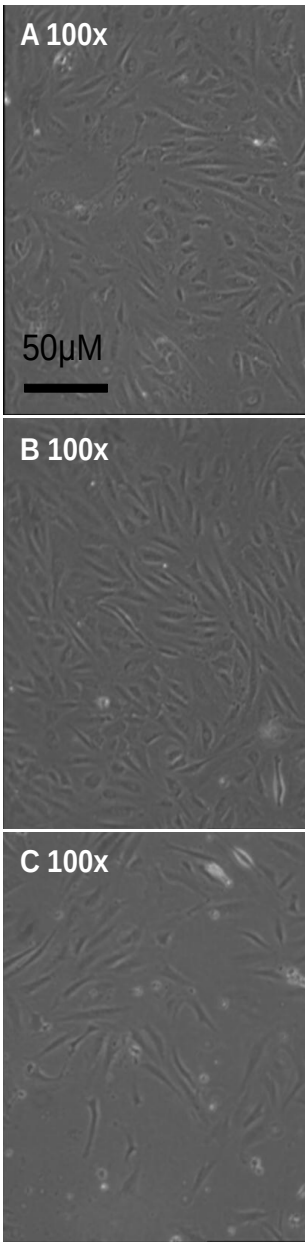


Figure 14: Morphological changes in HUVEC cultured in DL-Hcy, A: HUVEC grown in normal complete M199 media (5 days after passaging), B: HUVEC grown in complete M199 media containing 1mM DL-Hcy (5 days after passaging), C: HUVEC grown in complete M199 media containing 5mM DL-Hcy (5days after passaging). Arrows indicate elongated cells which are affected by high DL-Hcy treatment. (Original magnification: left column: 100x, right column: 200x.)

2.2.3.2. Characterization of chronic DL-Hcy effects on HUVEC viability

There have been previously published reports that 1mM DL-Hcy causes decreased EC viability (Lee *et al.*, 2005). Therefore, Trypan blue dye exclusion was used to distinguish between live and dead cells and this test was performed on cells without treatment and on those treated with 1mM DL-Hcy or Cys. The percentage of live cells was more than 95% in all treated and non-treated cells indicating that neither DL-Hcy nor Cys had toxic effects on HUVEC leading to reduced cell viability.

2.2.3.3. Characterization of chronic DL-Hcy effects on HUVEC proliferation

To evaluate the effects of DL-Hcy on HUVEC proliferation, the MTT assay was used. However, it was first necessary to determine the correlation between HUVEC cell number and the formed colour after carrying out the MTT assay and also to establish the best cell seeding density. HUVEC were seeded at different concentrations ($2.5x10^3$, $5x10^3$, $1x10^4$, $2x10^4$ and $4x10^4$ cells/well) in triplicate and incubated overnight. The MTT assay was then conducted and the relationship between cell number and absorbance was determined (Figure 15). The linear relationship demonstrated in Figure 15 shows that absorbance values are proportional to the number of the cells in each well. HUVEC were then seeded at two different densities, $1x10^4$ and $2x10^4$ cells per well in a 96-well plate and incubated for 96h. When a density of $1x10^4$ cells/well was used, there was a significant difference in the proliferation rate between non-treated cells and DL-Hcy-treated cells ($p<0.01$) (Figure 16) while no significant difference was observed between Cys-treated cells and non-treated cells. On the other hand, when a cell seeding density $2x10^4$ cells/well was used, there was no significant difference in the proliferation rate between either DL-Hcy or Cys compared to non-treated cells ($p>0.05$) (Figure 16). Furthermore, the MTT

assay was conducted on another set of cells every 24h for 4 days at the same densities used above ($1x10^4$ cells/well and $2x10^4$ cells/well). When the cell seeding density $1x10^4$ cells/well was used, results showed a significant difference in proliferation rate only on the fourth day (Figure 17) while no significant difference was observed at any time once $2x10^4$ cells/well seeding density was used. The percentile increase of cell numbers was calculated for each seeding density (Figure 18) and shows a daily increase in the cell numbers when $1x10^4$ cells/well seeding density was used. However, the percentage increase in cell numbers was lower once the highest seeding density was used.

Linear relationship between cell number and absorbance in the MTT assay

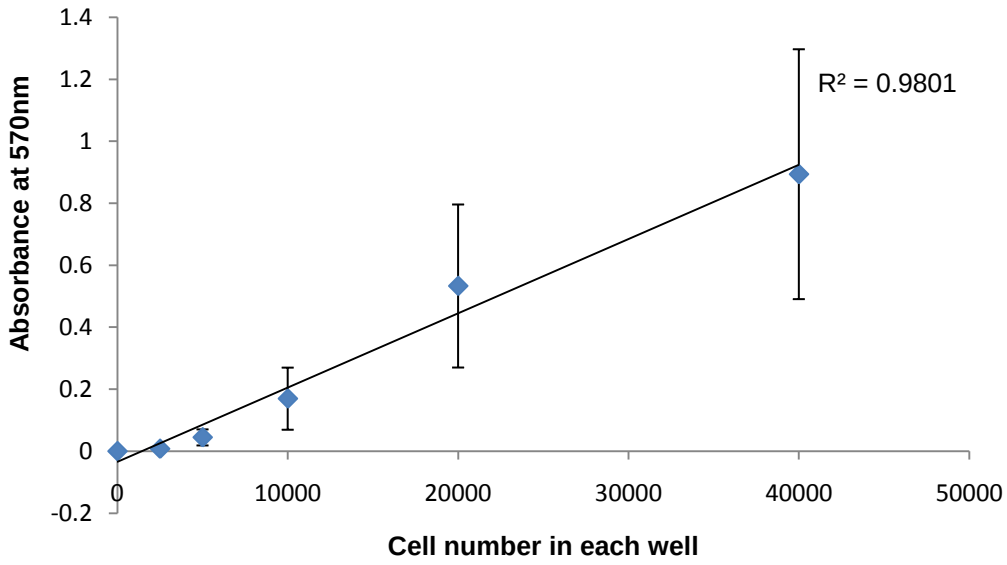


Figure 15: HUVEC cells were seeded in 96-well plate at different concentrations in triplicate and MTT assays were conducted after 24h. Linear relationship was observed between the numbers of the cells and the absorbance values. The results are expressed as mean (+/- SEM) from 3 different donors (n=3).

The effects of chronic exposure to DL-Hcy on cell proliferation rate

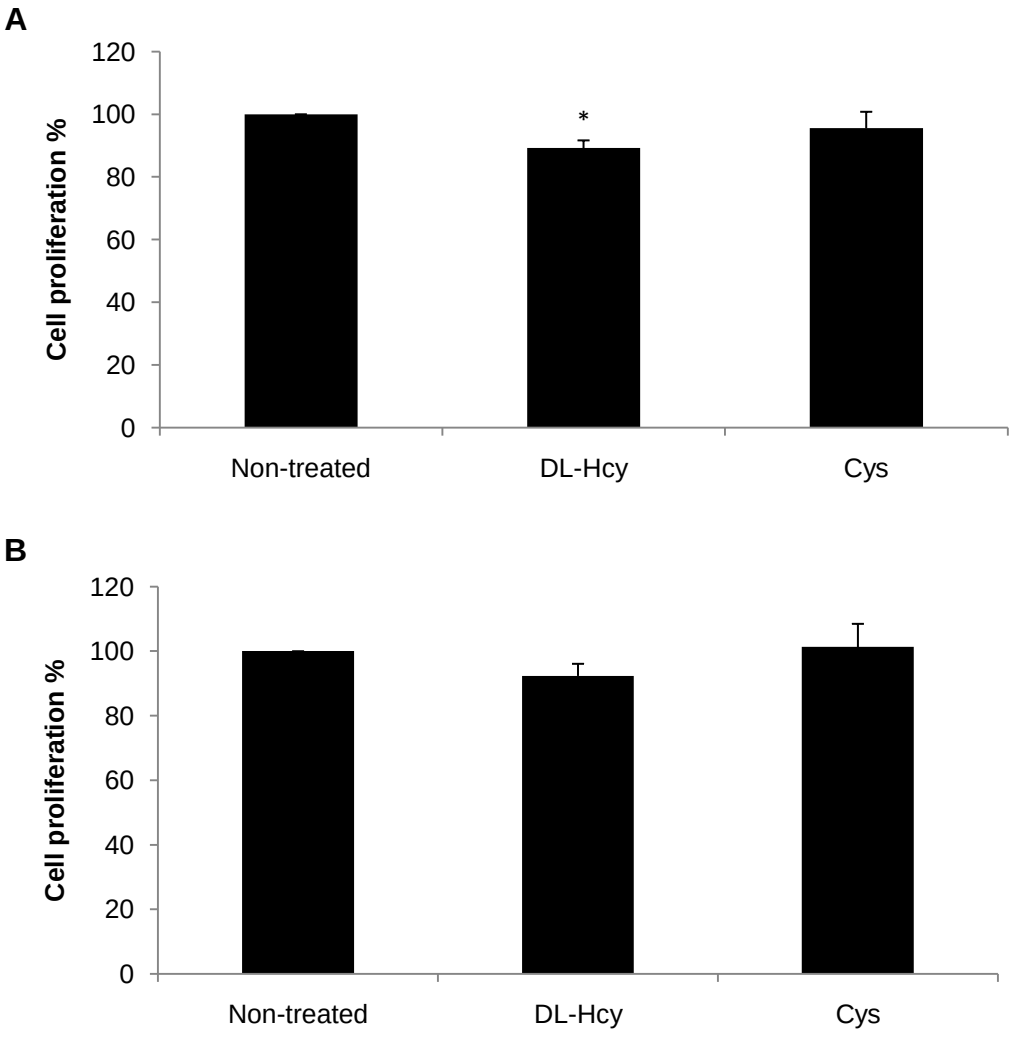


Figure 16: DL-Hcy (1mM) and Cys (1mM) -treated HUVEC cells were seeded in 96-well plate (A: $1x10^4$ cells/well and B: $2x10^4$ cells/well) in triplicate and MTT assays were conducted after 96h. Non-treated and Cys-treated HUVEC from the same donor were used in each experiment as negative controls. The results are expressed as mean (+/- SEM) from 9 different donors (n=9). Non-treated values were defined as 100% and other values were adjusted accordingly. (*) $p < 0.01$ for DL-Hcy versus non-treated cells.

The effects of DL-Hcy on cell proliferation rate over 4 days

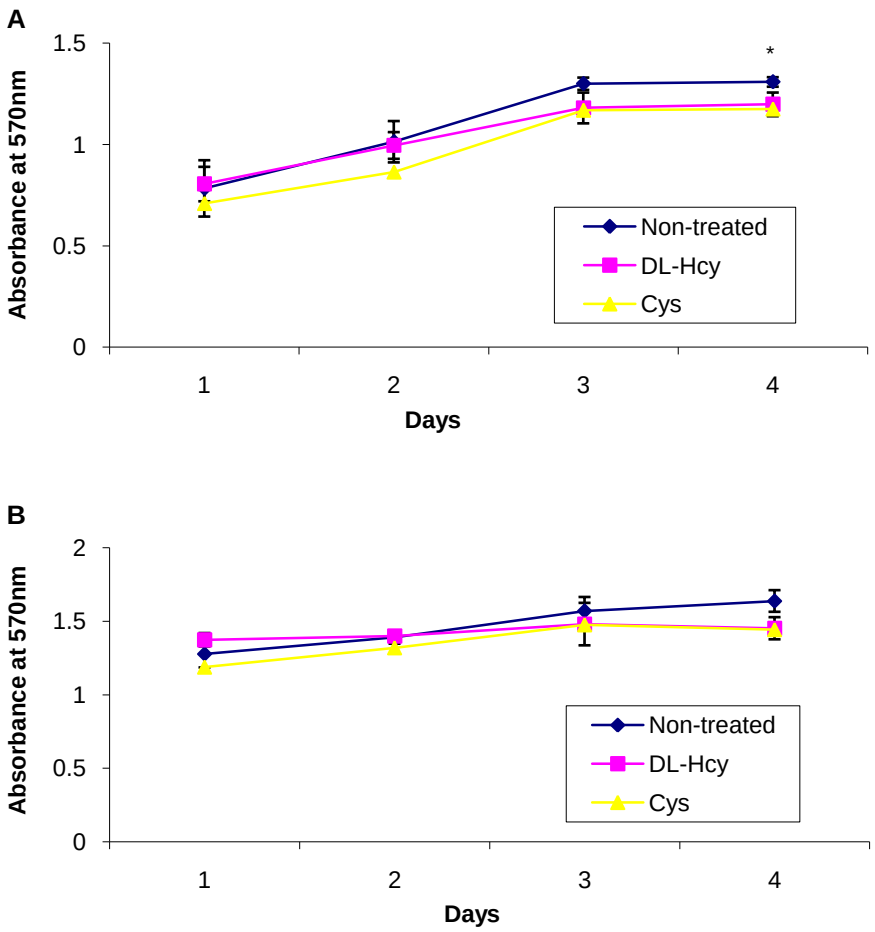


Figure 17: DL-Hcy-treated HUVEC (1mM) were seeded in 96 well plates (A: $1x10^4$ cells/well and B: $2x10^4$ cells/well) in triplicate and MTT assays were conducted every 24h for 4 days. Non-treated and Cys-treated (1mM) HUVEC from the same donor were used in each experiment as negative controls. The results are expressed as mean (+/- SEM) from 4 different donors (n=4). (*) $p < 0.05$ for DL-Hcy and non-treated cells.

The effects of chronic exposure to DL-Hcy on cell proliferation rate

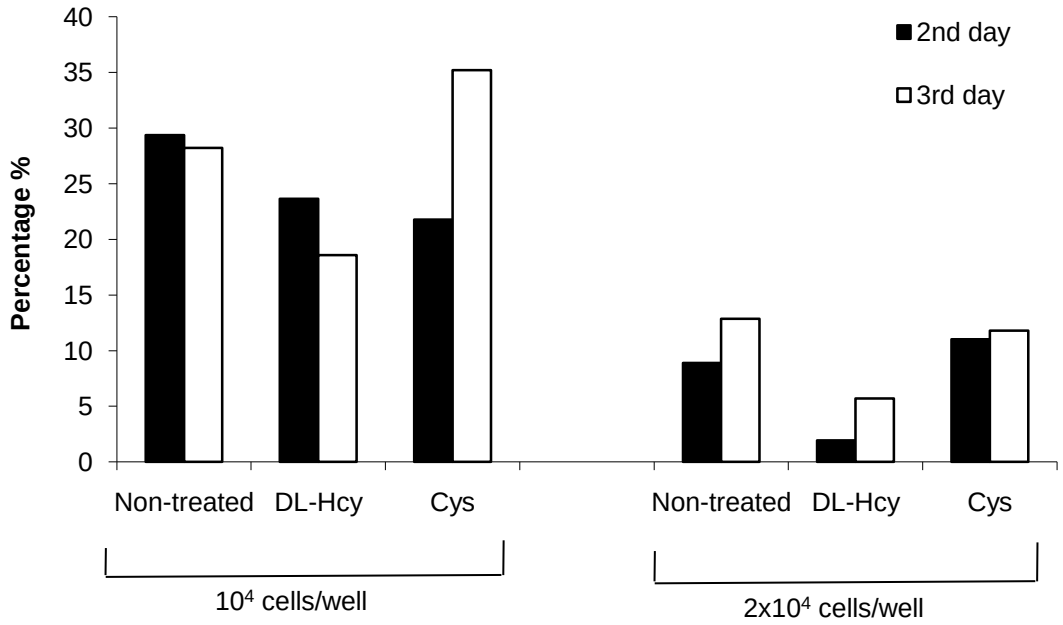


Figure 18: DL-Hcy -treated HUVEC (1mM) were seeded in 96-well plates ($1x10^4$ and $2x10^4$ cells/well) in triplicate and MTT assays were conducted every 24h for 4 days. Non-treated and Cys-treated HUVEC from the same donor were used in each experiment as negative controls. The results are expressed as percentile increases of the mean of the numbers of live cells every 24h from 4 different donors (n=4).

2.2.3.4. The effects of chronic exposure to DL-Hcy on the expression of cellular adhesion molecules

Using antibodies against ICAM-1, E-selectin and P-selectin, ELISA detected a slight increase in expression of these CAMs in response to DL-Hcy compared to non-treated cells. However, these increases were not significant (data not shown). Subsequently, immunofluorescence tagged antibodies against these CAMs were used to visualise their expression on the HUVEC surface. All three CAMs gave high fluorescence in response to DL-Hcy treatment compared to non-treated and Cys-treated cells (Figures 20, 21 and 22).

In DL-Hcy-treated cells both E-selectin and ICAM-1 showed cell surface localization in addition to paranuclear and Golgi localization (Figure 19). However, it was difficult to identify the localization of P-selectin in those cells. Therefore, histamine-treated cells were used to illustrate the localization of P-selectin after appropriate stimuli and Figure 19 shows that P-selectin was mainly localized in paranuclear locations in addition to showing cell surface expression.

Quantifying these results using ImageJ software (Figure 23) revealed that neither non-treated cells nor Cys-treated cells showed any significant expression for P-selectin or E-selectin. ICAM-1 expression was detectable in non-treated and Cys-treated cells. On the other hand, DL-Hcy induced significant expression of ICAM-1 and E-selectin compared to non-treated controls ($P<0.05$) but did not significantly increase P-selectin ($P>0.05$) on the HUVEC surface.

The effects of chronic exposure to DL-Hcy on CAM expression and their cellular localization

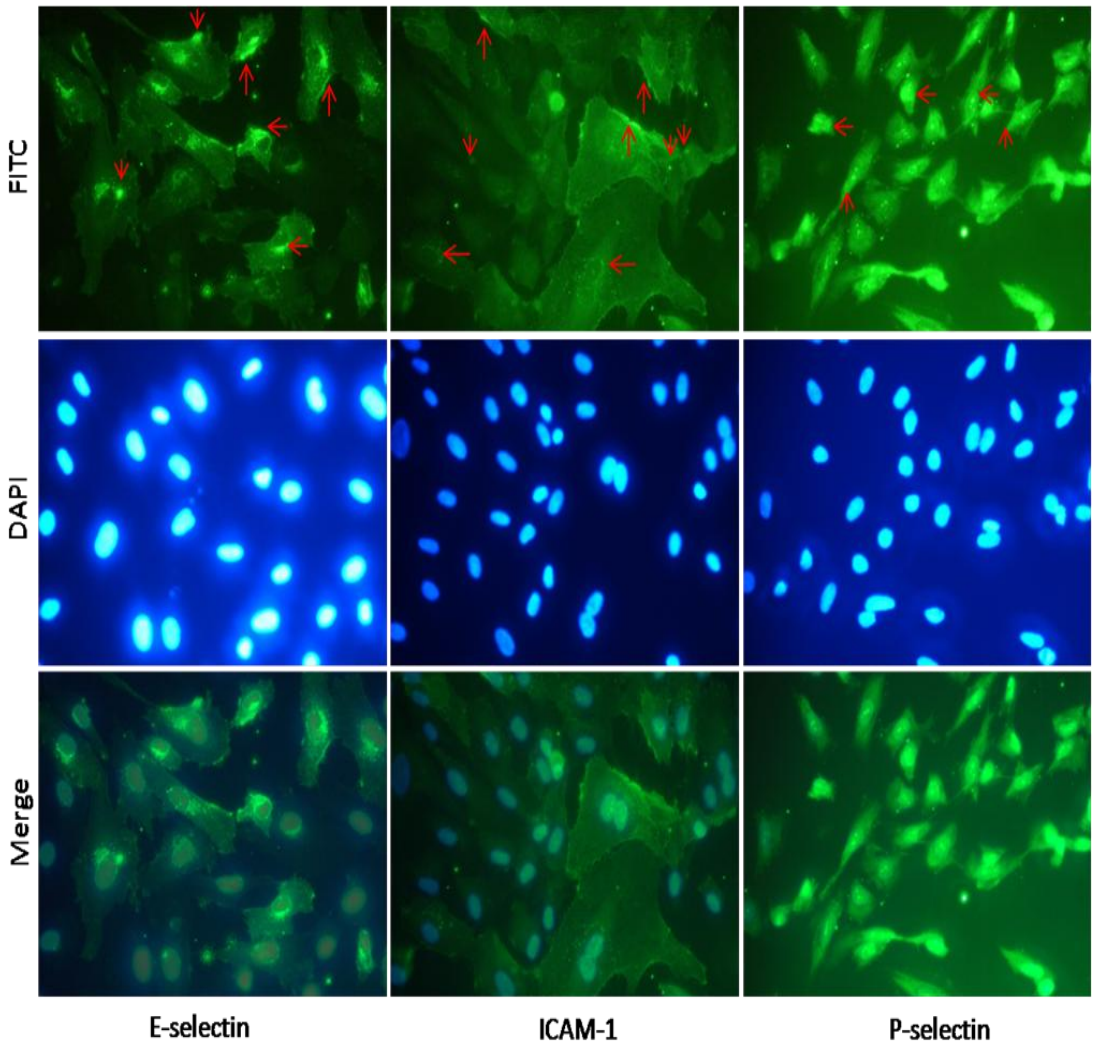


Figure 19: The cellular localization of each E-selectin, ICAM-1 after (1mM DL-Hcy, 5-9 days) and for P-selectin after (10^{-5}M histamine, 18min). The upper row is immunofluorescence microscopy detection of FITC, the middle raw is immunofluorescence microscopy detection of DAPI and the bottom one is merge of the two. The cellular localization of different molecules are indicated by arrows: (↑): cell surface localization, (↓): Golgi localization and (←): paranuclear localization.

The effects of chronic exposure to DL-Hcy on E-selectin expression

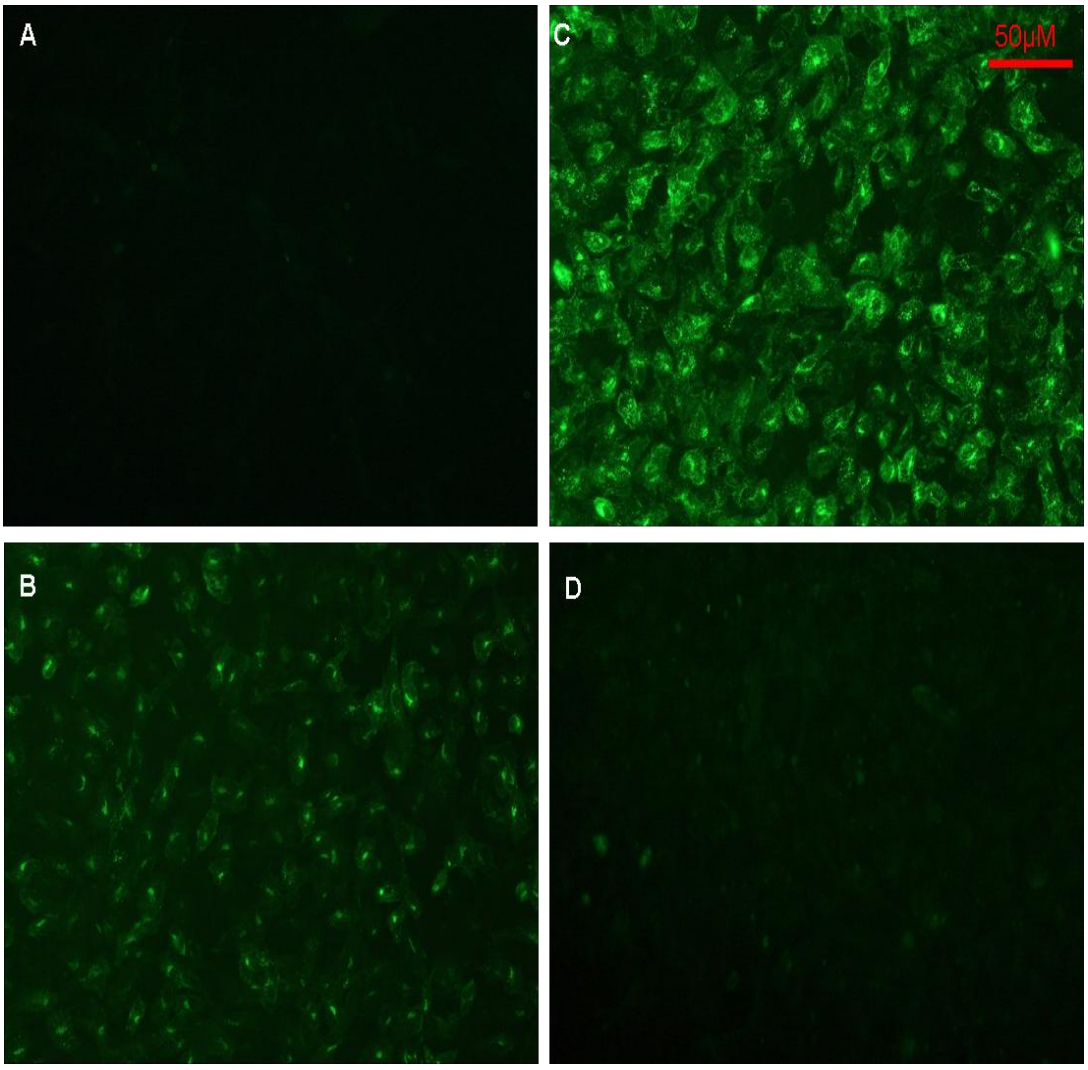


Figure 20: Representative images of the effect of DL-Hcy on E-selectin expression on HUVEC surface of 6 different experiments. HUVEC were grown in complete M199 medium and either left non-treated or treated with DL-Hcy (1mM; 5-9 days), Cys (1mM; 5-9 days) or TNF-α (120U/ml; 4h). Then, fixed cells were immunostained with FITC-tagged antibodies against E-selectin. A: non-treated HUVEC, B: DL-Hcy-treated HUVEC, C: TNF-α (120U/ml; 4h) treated HUVEC, D: Cys treated HUVEC. (Original magnification: 200x).

The effects of chronic exposure to DL-Hcy on ICAM-1 expression

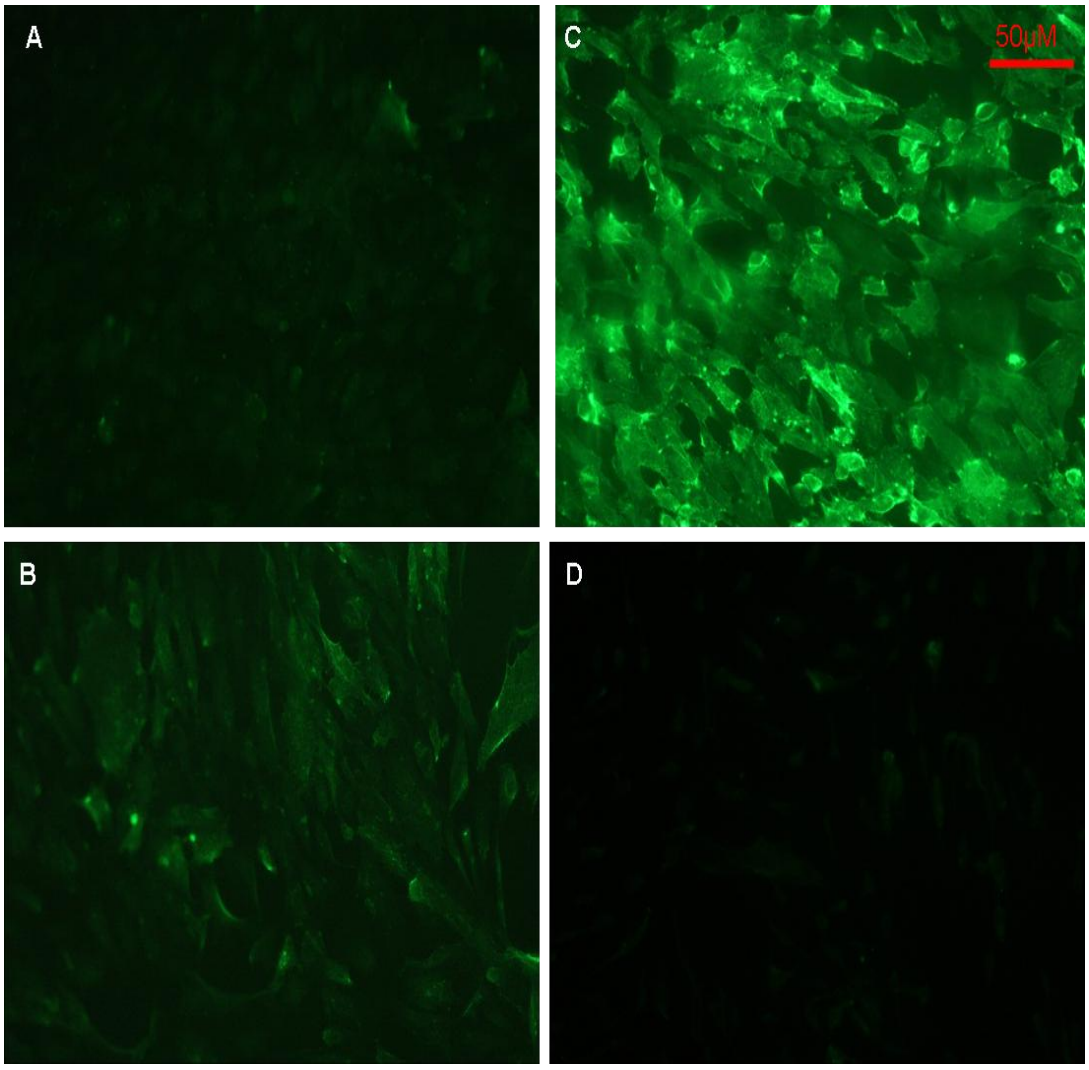


Figure 21: Representative images of the effect of DL-Hcy on ICAM-1 expression on HUVEC surface of 8 different experiments. HUVEC were grown in complete M199 medium and either left non-treated or treated with DL-Hcy (1mM; 5-9 days), Cys (1mM; 5-9 days) or TNF-α (120U/ml; 24h). Then, fixed cells were immunostained with FITC-tagged antibodies against ICAM-1. A: non-treated HUVEC, B: DL-Hcy-treated HUVEC, C: TNF-α (120U/ml; 24h) treated HUVEC, D: Cys treated HUVEC. (Original magnification: 200x).

The effects of chronic exposure to DL-Hcy on P-selectin expression

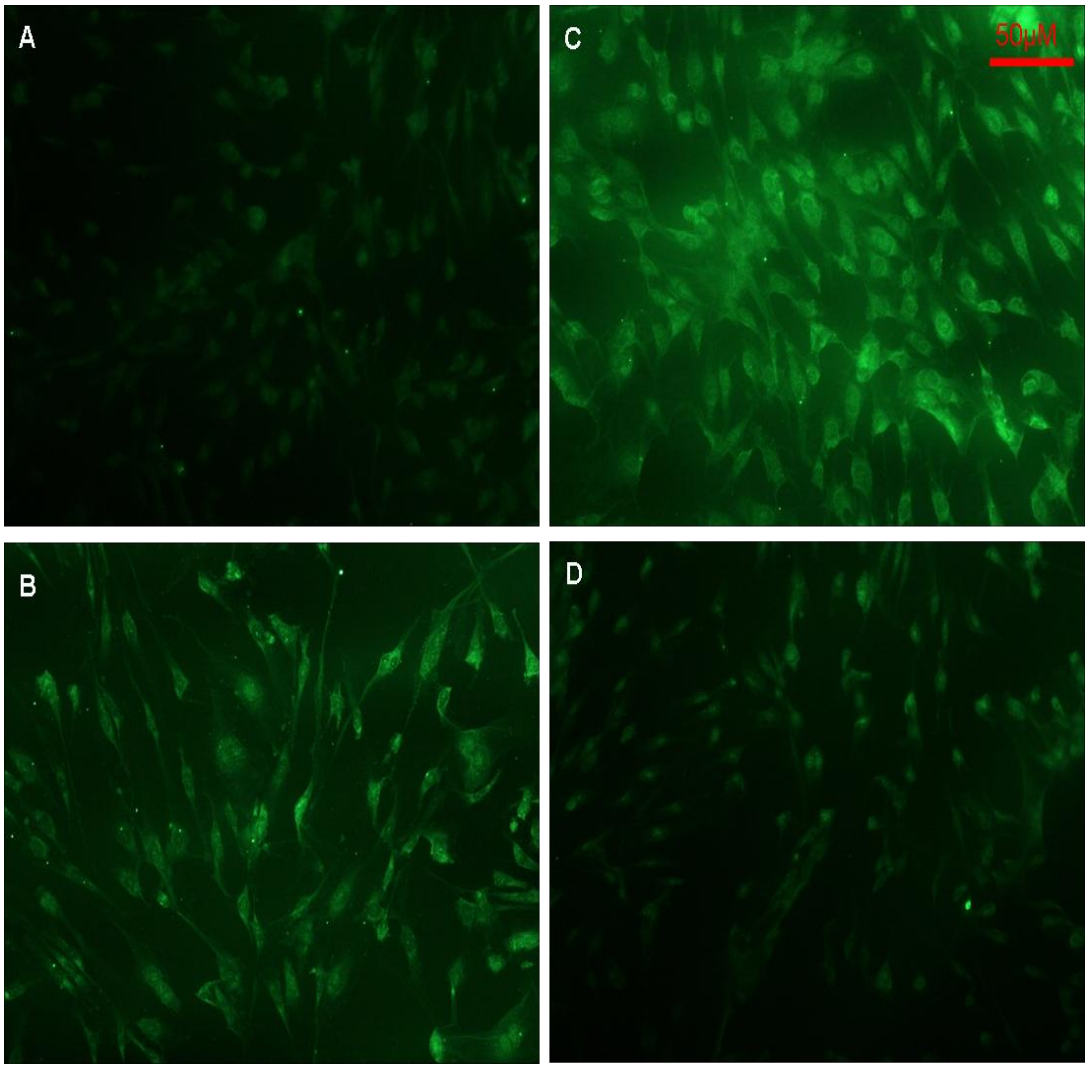


Figure 22: Representative images of the effect of DL-Hcy on P-selectin expression on HUVEC surface of 7 different experiments. HUVEC were grown in complete M199 medium and either left non-treated or treated with DL-Hcy (1mM; 5-9 days), Cys (1mM; 5-9 days) or histamine (10^{-5}M; 18min). Then, fixed cells were immunostained with FITC-tagged antibodies against P-selectin. A: non-treated HUVEC, B: DL-Hcy-treated HUVEC, C: histamine (10^{-5}M; 18min) treated HUVEC, D: Cys treated HUVEC. (Original magnification: 200x).

The effects of chronic exposure to DL-Hcy on CAM expression

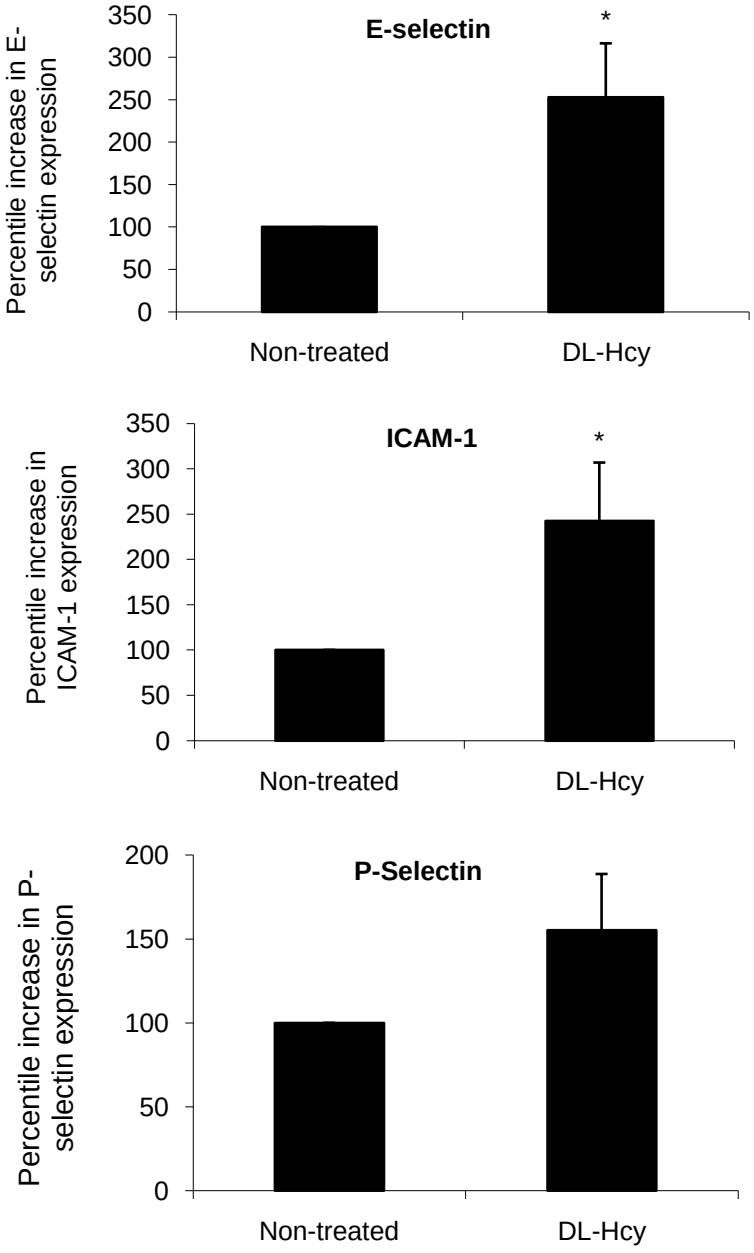


Figure 23: Fluorescence density of the immunostaining experiments of 4 randomly chosen fields for each experiment was quantified using ImageJ software. The results are expressed as mean (+/- SEM) from 6 different donors (E-selectin), 8 different donors (ICAM-1) and 7 different donors (P-selectin) for DL-Hcy and non-treated. Non-treated values were defined as 100% and other values were adjusted accordingly. (*) $p < 0.05$ for DL-Hcy versus non-treated cells.

2.2.3.5. Characterization of isolated neutrophils

2.2.3.5.1. Characterization of neutrophil viability

Neutrophil viability was tested using Trypan blue dye exclusion and cell viability was routinely found to be over 98%.

2.2.3.5.2. Characterizing the efficiency of the neutrophil isolation procedure

Neutrophils from each donor were counted and adjusted to the volume of donated blood and the range was 1.3-1.6 x 10^6 cells per ml of blood. Considering that the neutrophil count in a healthy person is 1.5 x 10^6 cells/ml, we can conclude that the efficiency of the isolation method was at least 94%.

2.2.3.6. The effects of chronic exposure to DL-Hcy on HUVEC:neutrophil interactions

EC were treated as described in section 2.1.2.2 and the effects of these treatments on the EC:neutrophil interactions are shown in Figure 24. The results are shown as mean number of interactions from 5 different experiments +/- SD. The numbers of all interactions in the negative controls remained negligible suggesting that the non-treated EC were not activated. However, cells which were cultured in complete M199 media containing 1mM DL-Hcy showed significant interactions. Both primary and secondary tethering interactions were significantly increased, as were the number of rolling, fixed and transmigrated neutrophils (p<0.05 in all cases). In contrast, stimulation with Cys had no effect on interactions and numbers were equal to or less than those for non-treated cells. Once these cells were treated with histamine (10^{-5}M; 18min), TNF-α (120U/ml; 4h) or TNF-α (120U/ml; 24h), all different sorts of interactions were induced and that was described previously by Eccles *et al* (2008). Figure 25 represents images taken from the flow experiments (CD containing movies of the flow experiments is included); Figure 25A and

B shows adhered and transmigrated neutrophils on DL-Hcy-treated HUVEC, while Figure 25C shows no neutrophils interacted with non-treated HUVEC.

The effects of chronic exposure to DL-Hcy on HUVEC:neutrophil interactions

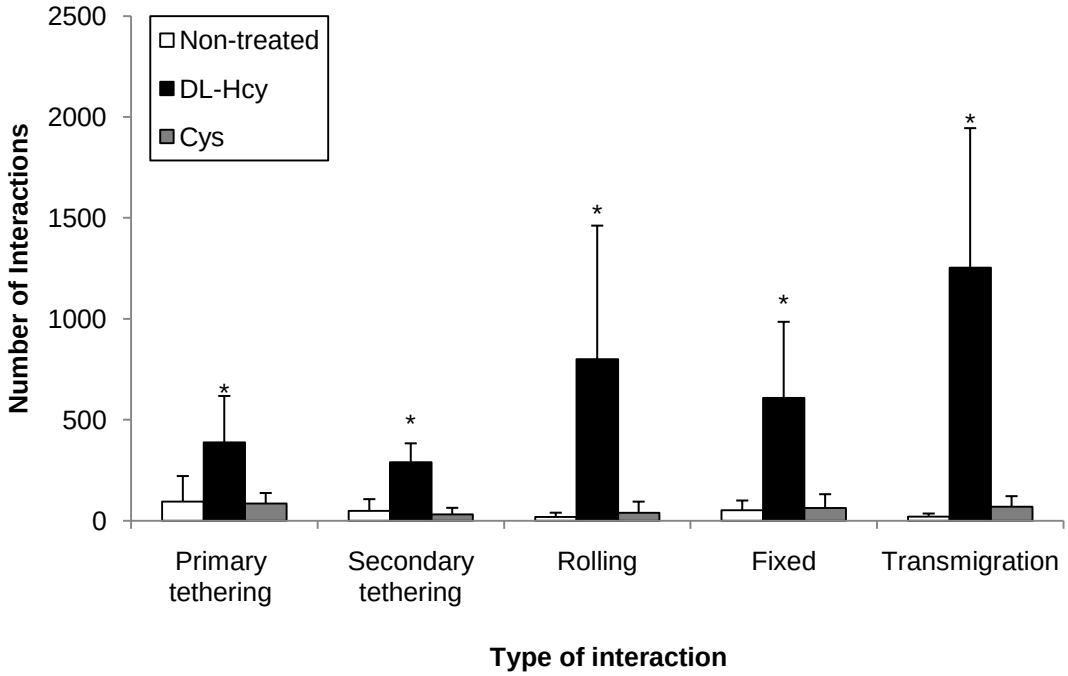


Figure 24: The effects of chronic exposure to DL-Hcy on HUVEC:neutrophil interactions. HUVEC were stimulated with DL-Hcy (1mM; 5-9 days), or Cys (1mM; 5-9 days). HUVEC from the same donor were grown in complete M199 media without any stimulation and considered with Cys-treated cells as negative controls. The effects on EC:neutrophil interactions under flow conditions were observed and quantified. The results are expressed as mean (+/- SD) from 5 different donors (n=5). (*) $p < 0.05$ for DL-Hcy and non-treated cells.

Representative images for the effects of DL-Hcy on HUVEC:neutrophil interactions under flow conditions

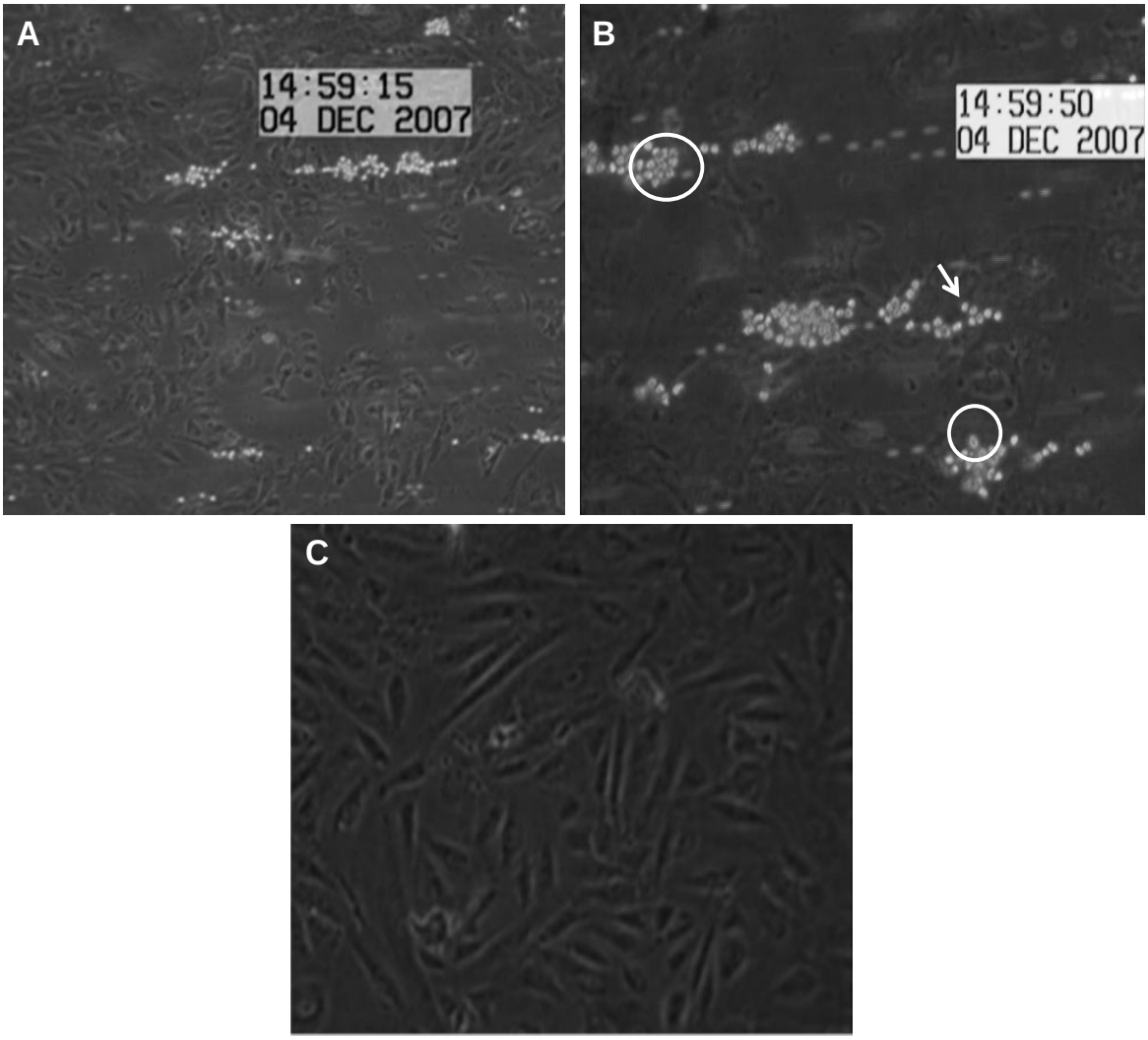


Figure 25: Representative images for the effects of DL-Hcy on HUVEC:neutrophil interactions under flow conditions of 5 different experiments. A (100x) and B (200x): neutrophils (white dots) adhered (arrow) and transmigrated (circle) through DL-Hcy-treated HUVEC. C (200x): non-treated HUVEC with no adhered neutrophils.

2.3. DISCUSSION

The inflammatory response is a series of local cellular and vascular responses to harmful stimuli, body injury, or bacterial infection. This inflammatory response includes the local vascular system, the immune system and various cells within the injured tissue (Galkina and Ley, 2009). A prolonged and uncontrolled inflammatory response (termed chronic inflammation) can develop into fatal diseases, e.g. atherosclerosis and autoimmune diseases. Atherosclerosis is defined as a chronic inflammatory response in the walls of arteries (Galkina and Ley, 2009; Lusis, 2000). Arterial EC represent the primary site for the initiation of atherosclerosis. Due to the fact that human arterial EC are not easy to obtain, different *in vitro* studies have used EC from a variety of sources to study the mechanisms behind atherosclerotic plaque formation (Dalal *et al.*, 2003; Ferretti *et al.*, 2004; Lee *et al.*, 2002; Zhang *et al.*, 2001a). HUVEC represent one easily accessible *in vitro* model to study atherosclerosis progression mechanisms and they have been used successfully in several studies (Dong *et al.*, 2005; Outinen *et al.*, 1999; Xu *et al.*, 2000). Even though HUVEC are venous EC, they still represent a more relevant model than murine or bovine arterial EC which have been used by some groups (Jin *et al.*, 2007; Weiss *et al.*, 2002). HUVEC are human EC which have been exposed to human blood containing the same growth factors, cytokines and other factors to which arterial EC are exposed.

Characterization of HUVEC isolated in this study showed that these cells retain important endothelial characteristics until at least passage number four as defined by adhesion molecule expression in response to classical acute inflammatory stimuli, staining with EC markers and growth characteristics and also by their basal expression of vWF and CD31 on their surface. Similar data has been previously published and it is likely that EC lose this

characteristic features once grown in culture for a longer time (Kaushal *et al.*, 2001). Since further characterisation had not been carried out on higher passage numbers, cells were not used beyond this number of passage in culture.

hHcy, which has a prevalence of 5-7% in the population, is considered an independent risk factor for cardiovascular disease (Lawrence de Koning *et al.*, 2003) (Section 1.1.5.4.1). It is therefore important to establish what effects high concentrations of Hcy have on EC function to determine its role in atherogenesis and atherosclerotic progression. Ranges of Hcy concentration that have been measured in clinical studies are not as high as those cultured cells have been exposed to. However, previous studies found that EC isolated from normal donors can adapt to stress conditions in culture and are relatively resistant to the effects of Hcy and require high concentration to induce intracellular uptake and to overcome cellular mechanisms responsible for Hcy metabolism and/or clearance (Dalal *et al.*, 2003; Hajjar, 1993; Outinen *et al.*, 1999; Rodgers and Kane, 1986). Therefore, EC in culture might require exposure to higher concentrations to increase intracellular uptake and overcome the cellular factors that influence the metabolism of Hcy (Hajjar, 2001; Werstuck *et al.*, 2001). In early experiments of this chapter, two different concentrations of DL-Hcy were used, 1mM and 5mM. The highest concentration significantly inhibited HUVEC proliferation in addition to changing their typical cobblestone shape (Figure 14) and these findings are consistent with those of Dalal *et al* (2003) who reported elongated shape of HSVEC after treatment with 5mM Hcy and also with Rodriguez-Nieto *et al* (2002) who showed that Hcy inhibits in a dose-dependent manner the proliferation of BAEC. It was decided not to carry out further experiments using this supra pathological concentration. Instead, 1mM DL-Hcy was used in all

subsequent experiments. Since the only commercially available Hcy is D/L mixture and only the L isomer is biologically active, the effective Hcy concentration was 0.5mM rather than 1mM. This was thought to be high enough to be pathologically relevant without being toxic to the cells because this concentration is within a similar range to patients with homozygous CBS deficiency. Most previous studies exposed cells to Hcy for only short periods (acute exposure; ≤ 24h). However, Hcy is a risk factor for chronic, rather than acute, inflammatory EC dysfunction. Therefore, in this chapter HUVEC were grown in the presence of DL-Hcy for at least one passage to ensure that all stages in the cell cycle during EC proliferation were exposed to the stressing agent.

Even though Hcy has been shown to alter different signaling pathways, its toxicity is still in debate and there is uncertainty whether activation of these pathways induces cell apoptosis or not. Therefore, this chapter initially determined effects on HUVEC viability and growth of 1mM DL-Hcy. Trypan blue exclusion results showed clearly that the concentration of DL-Hcy used in this study (1mM) was not cytotoxic to cells since the viability of DL-Hcy-treated cells was always greater than 95% with the number of dead cells similar to that observed in non-treated or Cys-treated cells. This finding are consistent with other studies which suggest that EC are resistant to Hcy cytotoxicity even at levels as high as 10mM (Outinen *et al* 1999), 5mM (Stangl *et al.*, 2001) or 3mM (Zhang *et al.*, 2001a). Although cell viability was not negatively affected, exposure to chronic DL-Hcy did slow down proliferation as measured by MTT. Firstly, a linear relation between cell numbers and absorbance of formed formazan was established (Figure 15) which means the absorbance values were proportional to the number of live cells and therefore demonstrates that the MTT results reflect cellular proliferation rate. Two different seeding densities were used in

the MTT assay ($1x10^4$ and $2x10^4$ cells/well). The data in Figure 16 shows that DL-Hcy significantly decreased the proliferation rate of HUVEC seeded at the lowest density. The measurement of proliferation rate after 24h showed that there was no difference between non-treated and DL-Hcy-treated cells until the 4^{th} day of DL-Hcy treatment when cells were seeded at $1x10^4$ cells/well (Figure 17). This highlights the fact that in hHcy patients, where EC are exposed to high levels of Hcy for years, EC proliferation may be reduced compared to EC in normal people. EC proliferate at a low rate normally but start proliferating quickly in response to a lesion or in angiogensis. Therefore, it could be said this Hcy-induced slow in proliferation might accelerate lesion development. This data indicates changes in gene expression that might make cells less responsive to growth factors. That might make the cells more primed for atherosclerosis and could be responsible for other Hcy-induced effects. When higher numbers of HUVEC were used to assess proliferation ($2x10^4$ cells/well), the difference between proliferation rate of DL-Hcy-treated and non-treated cells was not significant (Figure 16). Plating this cell density allowed them to reach confluence in each well prior to day 4 resulting in their proliferation rate being suppressed by contact inhibition (Figure 18). Therefore, non-treated and DL-Hcy-treated cells, at such high plating density, might have reached confluence within less than 96h and thus ended up with no difference between numbers of live cells after 96h. When fewer cells were plated ($1x10^4$ cells/well) a significant reduction in proliferation in response to DL-Hcy was recorded after 96h. On the other hand, Cys did not affect HUVEC proliferation and their proliferation rate was similar to non-treated cells. Mitochondrial dehydrogenase of viable cells converts the tetrazolium into formazan which has a purple colour and the intensity of the colour is proportional to the number of live cells. However, this enzyme

could be altered by Hcy treatment and therefore, that might result in inaccurate results. Therefore, it might have been useful to measure the proliferation rate with other techniques in addition to the MTT such as [^{3}H]thymidine incorporation or the inhibition of expressions of gene which are known to be involved in regulating cell cycle (Wang *et al.*, 2002; Wang *et al.*, 1997).

These findings are in line with the findings of other groups like Nagai et al who found that Hcy (within 5 days) inhibited the proliferation of bovine aortic endothelial cells (BAECs) in a dose-dependent manner and there was almost no cell proliferation in the presence of 10mM homocysteine (Nagai *et al.*, 2001). Similar to the results with Cys-treated cells in this chapter, the proliferation of BAECs was not inhibited by cysteine. Other studies showed also that treatment with Hcy for 24h, even at the low concentration of 20µM, decreases [^{3}H]thymidine incorporation into HUVEC by 50% (Wang *et al.*, 2002; Wang *et al.*, 1997) and the cell line SVEC4-10 (mouse lymph node endothelial cell line) (Ohashi *et al.*, 2006), which supports the results in this chapter showing that Hcy has inhibitory effects on EC proliferation. Wang's group found that mRNA expression and protein levels of Cyclin A, which is a key regulatory cyclin regulating G1 and G1/S transition of cell cycle, were significantly decreased by both homocystine and Hcy (25-50µM/30h). They also showed that overexpression of cyclin A was sufficient to overcome the G1/S phase block caused by Hcy. They believe that suppression of this cyclin was responsible for Hcy-induced cell growth arrest and suggested these effects to happen in very late G1 or at the G1/S transition (Wang *et al.*, 2002). Furthermore, Hcy was reported to have cell-type specific proatherogenic effects as it suppresses EC growth (the results of

the current chapter), Ohashi *et al* (2006) and Wang *et al* (1997), while inducing VSMC proliferation (Tsai *et al.*, 1994).

Endothelial activation has been proposed to be one of the initiating events of atherosclerosis and it is characterized by the induction of several intracellular signaling pathways leading to up-regulation of several proteins like CAMs which will be mobilized and expressed on the activated EC surface (Galkina and Ley, 2009; Lusis, 2000). To investigate whether DL-Hcy induces EC activation, specific monoclonal antibodies against ICAM-1, E-selectin and P-selectin were used to detect the expression of these CAMs on the EC surface in response to chronic treatment with DL-Hcy under static conditions. Firstly, an established ELISA technique was used. However, the results were not encouraging and no significant CAM expression was detected even after stimulation with proinflammatory mediators as positive controls (histamine and TNF-α). The antibodies which were used in the ELISA were capture antibodies that detect soluble proteins. Despite the fact these soluble proteins should be similar to surface expressed adhesion molecules, it was difficult to explain how these capture antibodies were not able to detect the surface expressed CAM. As an alternative experimental approach, HUVEC were immunostained with FITC-tagged antibodies against these CAM to directly visualize alterations in expression. Both ICAM-1 and E-selectin showed significant up-regulation of expression in DL-Hcy-treated cells compared to non-treated cells or Cys-treated cells (Figures 20, 21 and 23). Furthermore, both molecules showed in addition to cell surface localization, paranuclear and Golgi localization which indicate *de novo* synthesis of these two molecules (Figure 19) and that means after 5-9 days treatment with DL-Hcy, E-selectin and ICAM-1 synthesis was still going on in addition to sustained cell surface expression. Collectively that means the cycle

of synthesizing and mobilizing these molecules to cell surface was taking place as long as the DL-Hcy was present. ICAM-1 was detectable in non-treated and Cys-treated cells because it is well known that HUVEC express ICAM-1 under basal conditions. Even though P-selectin expression on DL-Hcy-treated EC appeared to increase as detected by eye (Figure 22), the increase was not quite statistically significant (Figure 23). Localization of P-selectin in DL-Hcy-treated cells was difficult to study as the staining was not strong enough. Therefore, positive control cells were used in Figure 19 to illustrate P-selectin localization and this CAM showed in addition to the cell surface staining mainly paranuclear localization which is typical for P-selectin as this adhesion molecule is usually stored in WPB (McEver *et al.*, 1989) before being mobilized to the cell surface.

Previous studies have demonstrated that acute exposure to Hcy induced expression of P-selectin in murine venular EC (5mM DL-Hcy/150min) (Pruefer *et al.*, 1999), E-selectin in HAEC (100µM/20h) (Koga *et al.*, 2002) and ICAM-1 in HUVEC and in murine venular EC (200µM/6h and 5mM DL-Hcy/150min) (Postea *et al.*, 2006; Pruefer *et al.*, 1999). Although the findings of this chapter do not totally agree with Pruefer *et al* (1999), this may well reflect differences between responses of murine endothelium and HUVEC or that acute effects may be different from chronic effects and might induce increases in the expression of the CAM which then decreases to basal levels. Furthermore, Mansoor *et al* found after methionine loading in healthy subjects, increased plasma levels of circulating ICAM-1 and E-selectin and to lesser extent P-selectin (Mansoor *et al.*, 2004). Their results correlate with the immunostaining results of this chapter except that they studied the soluble form of these CAMs while this chapter investigated the surface expressed CAM. It has been described previously that cytokines induce peak expression of surface E-selectin

followed by decrease which was associated with a peak of soluble E-selectin (Wyble *et al.*, 1997). This means that the only source of soluble CAM is the shed receptors and in the case of chronic Hcy treatment, the CAM synthesis pathway might be continuously activated which leads to replacement of the shed receptors. On the other hand, some other studies have found that acute treatment with Hcy (5mM/15min) was not able to induce significant expression of CAM in HUVEC (Stangl *et al.*, 2001) and that could be explained by the short incubation time. Furthermore, Dudman and Hale (1997) and Dudman *et al* (1999) found that 200µM Hcy was not able to induce E-selectin, ICAM-1, or P-selectin in HUVEC. Dudman's group co-cultured neutrophils (indirectly) and HUVEC and treated the neutrophils with Hcy assuming that Hcy might induce them to release cytokines which might induce CAM up-regulation on HUVEC. However, this treatment failed to induce any expression of CAM on HUVEC (Dudman *et al.*, 1999).

Most of the previous studies which investigated EC:leukocyte interaction and the expression of CAMs were conducted under static conditions (Dudman *et al.*, 1999; Lin *et al.*, 2008; Postea *et al.*, 2006; Silverman *et al.*, 2002). Static studies results are not particularly relevant to the interactions in the blood vessels because they don't consider the flow forces and shear stress. Therefore, the *in vitro* flow model, which was used in this study, made it easy to study the different sorts of interactions between the two cell types under conditions which represent binding with discrete adhesion molecules e.g. tethering with P-selectin and rolling with E-selectin. The *in vitro* flow model allows the study of the expression of these CAM under flow conditions through studying different sorts of interactions between HUVEC and neutrophils which are mediated by these adhesion molecules in real time.

Neutrophils, which represent approximately 60% of all leukocytes, are widely used in different *in vitro* models to study inflammatory responses (Dudman *et al.*, 1999; Jones *et al.*, 1996; Woolhouse *et al.*, 2005). Furthermore, adhesion of neutrophils to EC, as well as being the main event in acute inflammation, has also been shown to play a role in early atherosclerotic lesion development (Jia *et al.*, 2005; Zernecke *et al.*, 2008). While it is accepted that adhesion of monocytes and lymphocytes to EC appears to be important in the later stages including the formation of fatty streaks (reviewed by Lusis (2000)), this study focuses on roles of Hcy in initiation of atherosclerosis and thus neutrophils were used in the *in vitro* model.

The data in Figure 24 demonstrate that DL-Hcy induced up-regulation of EC:neutrophil interactions under flow conditions: tethering, rolling, adhesion and transmigration ($p<0.05$ in all cases). Mayadas *et al* (1993) have previously demonstrated in genetically modified mice that P-selectin, on the level of EC, is the main mediator of the first contact between leukocyte and EC which was later termed tethering. However, the results from the static immunostaining experiments in this chapter did not give evidence of significant DL-Hcy-induced P-selectin up-regulation. Despite this, the number of tethered neutrophils on DL-Hcy-treated HUVEC as quantified in the flow model was significantly higher than that of non-treated or Cys-treated cells. Collectively, it could be concluded that DL-Hcy up-regulated P-selectin expression to a level high enough to mediate tethering between neutrophils and HUVEC and thus initiate the first step in the inflammatory response or atherosclerotic plaque formation. P-selectin density on activated EC *in vivo* is still unknown but thought to be much lower than E-selectin on also on the activated platelets surface (less than 10,000 per cell) (Blann *et al.*, 2003). This level of expression

could be below the limit of detection for both ELISA and immunostaining and might explain why these technique used on static cells was not able to reveal significant expression of P-selectin. This data are in line with several previous studies which also failed to detect a significant expression of soluble P-selectin by HUVEC (Blann *et al.*, 2003). However, another study showed that Hcy induced up-regulation of both soluble P-selectin and in aortic sections of hHcy mice (CBS deficient) (Weiss *et al.*, 2002). This study used arterial EC which might express P-selectin at a higher level than venous EC which were used in this study. Collectively, these data suggest that it is difficult to detect P-selectin even after appropriate stimulation. However, the flow model provided strong indirect evidence of DL-Hcy-induced P-selectin up-regulation (Figure 24). One study suggested that P-selectin can mediate rolling (Lawrence and Springer, 1991). However, this study was conducted on artificial lipid membranes containing different levels of immobilized E-selectin and P-selectin. Our lab has previously investigated role of P-selectin in Hcy-mediated response under flow using specific blocking P-selectin antibodies and shown that blocking P-selectin inhibited tethering without affecting rolling and the following stages which means P-selectin is the main mediator of tethering (unpublished data). Immunostaining experiments in this chapter revealed that DL-Hcy induced significant expression of E-selectin ($p<0.05$). Furthermore, under flow conditions, the number of rolling neutrophils on DL-Hcy-treated HUVEC significantly increased which suggests that DL-Hcy treatment induced EC activation resulting in expression of the specific CAMs which mediate neutrophil rolling. Several studies have shown that E-selectin is the main mediator of leukocyte rolling on activated EC and its level is negatively correlated with rolling velocity (Abbassi *et al.*, 1993; Jung and Ley, 1999; Kunkel *et al.*,

2000). Therefore, the significant increase in the number of rolling neutrophils in the flow model reflects an up-regulation of E-selectin expression under flow conditions. Therefore, the experiments in this chapter suggest that DL-Hcy-induced continued up-regulation of E-selectin was responsible for mediating neutrophil rolling on EC.

Immunostaining experiments gave strong evidence that DL-Hcy induces ICAM-1 expression on HUVEC surface ($p<0.05$ compared to non-treated cells). It has been shown previously that members of the immunoglobulin superfamily, like ICAM-1 (Sligh *et al.*, 1993) and VCAM-1 (Carlos and Harlan, 1994) act as receptors for integrins on the leukocyte surface to mediate the firm adherence of leukocytes to EC. The results from the flow experiments strongly support these findings and collectively with the immunostaining results, it could be said that DL-Hcy induced ICAM-1 expression under static conditions. ICAM-1 expression was high enough to mediate the firm adhesion of neutrophils to HUVEC under flow conditions. On the other hand and despite the basal expression of ICAM-1 by non-treated cells which is seen in the immunostaining images (Figure 21), that was not enough to induce significant EC:neutrophil interactions under physiologically relevant flow conditions.

Several previous studies have been conducted to investigate the effects of Hcy on EC:leukocyte interactions. An *in vivo* study on murine vein using intravital microscopy demonstrated that 1mM Hcy induced significant increases in leukocyte rolling within 1h of treatment (Pruefer *et al.*, 1999). Despite their finding about CAM, Dudman's group found that Hcy treatment induced enhanced neutrophil endothelial interactions in cultured human cells and in rat venules *in vivo* but they believe Hcy induces HUVEC to release appropriate cytokines like IL-8 and MCP-1 which will mediate such interactions (Dudman *et al.*, 1999).

Hcy was shown also to enhance HAEC adhesion to activated mononuclear cells from patients with coronary artery diseases (Lin *et al.*, 2008). Furthermore, treatment with 200μM Hcy for 6h induced significant increases in monocyte adhesion to EC cell line (EA.hy 926) (Postea *et al.*, 2006). Monocytes were also shown to adhere to Hcy-treated HAEC (100μM/18h) under static conditions and they demonstrated that VCAM-1 and ICAM-1 were involved in this step (Silverman *et al.*, 2002). Dudman's group demonstrated also that neutrophils adhered to Hcy-treated HUVEC. Furthermore, these neutrophils managed to migrate through the monolayer of HUVEC (Dudman *et al.*, 1999).

All of the above mentioned studies are consistent with the findings of this chapter although most of them were conducted under static conditions and studied only one type of EC:leukocyte interaction. Therefore, they do not provide a complete picture of what happens in humans with hHcy. Therefore, this study is the first to show that chronic exposure to DL-Hcy alone induces continuous up-regulation of these three CAMs up to 9 days and also induces all types of interactions between DL-Hcy-treated EC and leukocytes under flow conditions in the absence of any additional cytokine stimulation. It is possible that chronic treatment with DL-Hcy will generate and maintain proinflammatory conditions by inducing the production of proinflammatory cytokines and that may augment the effects of DL-Hcy on CAM expression and leukocyte interaction with EC. These findings are different to the findings of pro-inflammatory cytokine effects on CAM expression e.g. cytokine-induced up-regulation of E-selectin which returns to basal within 24h (Bevilacqua *et al.*, 1989) or histamine-induced up-regulation of P-selectin which peaks within 20min (McEver *et al.*, 1989) then return to basal levels demonstrating differences in EC responses to chronic inflammatory mediator Hcy compared to TNF-α/histamine.

All the experiments conducted in this chapter found that results of Cys-treated cells were similar to non-treated cells which means that all of the observed effects with Hcy-treated cells were specific to Hcy and were not reproducible with its metabolite Cys. These findings match with previous studies which confirmed the unique properties of Hcy-induced EC damage (Carmel and Jacobsen, 2001; Jacobsen, 2000; Sengupta *et al.*, 2001; Wang *et al.*, 1997). This data also confirms that the relatively high Hcy concentrations are not affecting the cells through osmotic/nonspecific mechanisms.

In summary, the static *in vitro* experiments showed that DL-Hcy-induced significant expression of E-selectin and ICAM-1 and to a lesser extent P-selectin on HUVEC cell surfaces. The direct demonstration of up-regulation is further enhanced by results in the flow model showing significant tethering (P-selectin), rolling (E-selectin) and firm adhesion (ICAM-1) in response to DL-Hcy. Collectively, chronic treatment with DL-Hcy had several vital consequences in EC. It significantly deceased proliferation rate and induced changes to genetic expression causing up-regulation of different CAM. These CAM in turn mediated different sorts of interactions between EC and leukocytes which represent the first step in atherosclerosis plaque formation.

2.4. CONCLUSION

Chronic exposure to high levels of DL-Hcy (1mM) did not kill HUVEC in culture. However, it caused a decrease in cellular proliferation rate. Furthermore, DL-Hcy induced changes in cell morphology and induced sustained gene expression in the EC which led to up-regulation of CAM as demonstrated both statically (immunofluorescence) and under flow. These DL-Hcy-induced CAM mediated the recruitment of leukocytes to the activated

EC and promoted significant interactions between the two cell types which can be considered to represent the first step in atherosclerosis lesion initiation.

CHAPTER 3

3. JNK ROLE IN HCY-MEDIATED EC ACTIVATION

EC activation has different consequences and might lead to cell apoptosis and/or cell involvement in inflammatory interactions. JNK involvement in this activation and cellular effects has been studied intensively. Various previous studies reviewed by Sumara *et al* (2005) reported a role for JNK in mediating TNF-α-induced apoptosis in different cell types. The downstream effects of JNK pathway activation includes phosphorylation of a number of transcription factors, including the c-Jun component of the activation protein-1 (AP-1) transcription factor family (Sumara *et al.*, 2005) in addition to other AP-1 proteins, including JunB, JunD and ATF2 (Davis, 2000). These transcription factors have functional binding sites on different genes including ICAM-1 (Voraberger *et al.*, 1991), E-selectin (Hoefen and Berk, 2002; Min and Pober, 1997), VCAM-1 (Ahmad *et al.*, 1998) and IL-8 (Roebuck, 1999). All the products of these previous genes are reported to be involved in mediating interactions between leukocytes and endothelial cells and hence mediating the inflammatory response. This highlights the key role of JNK in inflammation and thus potentially in the chronic inflammatory disease atherosclerosis. Further evidence comes from experiments with knocked down JNK which induced up-regulation of other MAPK-mediated pathways such as ERK1/2 (Hoefen and Berk, 2002) or other pathways involved in the development of atherosclerosis (Ricci *et al.*, 2004).

Chapter 2 indicates clearly that Hcy induced EC activation characterized by up-regulation of several adhesion molecules in addition to increased numbers of HUVEC:neutrophil interactions. However, the signalling pathway through which Hcy

induces such effects is still unclear. Previously published work in cultured EC demonstrated that acute Hcy exposure induced activation of the stress activated MAP kinase family member JNK and its downstream substrate c-Jun (Dong *et al.*, 2005; Zhang *et al.*, 2001b). Therefore, this chapter aimed to demonstrate whether chronic treatment with Hcy induces sustained activation of the JNK pathway and whether this pathway is responsible for the up-regulation of CAM already demonstrated in Chapter 2 under static and flow conditions.

3.1. MATERIALS and METHODS

3.1.1. Materials

3.1.1.1. Reagents

All reagents used for SDS-PAGE and western blotting were of electrophoresis grade. Sodium dodecyl sulfate (SDS, L3771), glycine (G-8898), sodium pyrophosphate (S-9515), bromophenol blue (B-5525), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, T-9281), DL-dithiothreitol (D-0632), polyoxyethylene-sorbitan monolaurate (Tween 20, P-1397), bovine serum albumin (BSA, A-4503), ammonium persulfate (APS, A-3678), 1,9-Pyrazoloanthrone (SP600125, S5567), Kodak GBX developer and replenisher (P7167) and Kodak fixer and replenisher (P7042), were all purchased from Sigma-Aldrich (Poole Dorset, UK). Acrylamide/Bis Solution (30%) was obtained from BioRad Labs (161.0156, Bio-Rad Laboratories, Hercules, California, USA). X-ray film was obtained from Amersham (Amersham hyperfilm ECL, RPN3114K) and PVDF membrane (Hybond-P, RPN303F) was purchased from GE healthcare, Buckinghamshire, UK. Reagents for enhanced chemiluminescence (ECL) were purchased from Amersham Life Sciences, Little Chalfont, Bucks, UK.

3.1.1.2. Antibodies

Mouse monoclonal antibodies against phospho-SAPK/JNK (catalog No. 9255) were purchased from Cell Signaling Technology Inc, Danvers, MA, USA. Rabbit polyclonal antibodies against phospho-SAPK/JNK (catalog No. V7931) were obtained from Promega, Southampton, UK. ECL anti rabbit IgG horseradish peroxidase linked whole antibody (NA934V) and ECL anti mouse IgG horseradish peroxidase linked whole antibody (NA931V) were obtained from GE healthcare UK limited, Buckinghamshire, UK.

Prestained protein marker broad range (P7707S) was purchased from New England Biolabs. Biotinylated protein ladder (catalog No. 7727) and HRP conjugated anti biotin antibodies (catalog No. 7075) were purchased from Cell Signalling Technology. The diluent which has been used to dilute all antibodies was TBS-T including 2% w/v BSA.

3.1.1.3. JNK inhibition

SP600125 was used to inhibit the activity of phosphorylated JNK. It was added to the media with the DL-Hcy to a final concentration of 3μM, which has been previously shown to selectively inhibit JNK without affecting any other MAPK family members (Bennett *et al.*, 2001). Media was changed every 24h during the treatment with SP600125 to avoid any possible degradation of the inhibitor.

3.1.2. Methods

3.1.2.1. Detection of phospho-JNK by sodium dodecylsuphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

3.1.2.1.1. Preparation of samples for analysis by SDS-PAGE

Following the appropriate stimulation, the reaction was stopped by placing the plates containing cells on ice and rinsing twice with ice-cold HPFEV buffer (50mM Hepes, 10mM sodium pyrophosphate, 100mM sodium fluoride, 4mM EDTA, 2mM orthovanadate). Integrity of the confluent monolayers was confirmed by microscopic observation. 300μl of hot (65°C) Laemmli buffer (0.048M Tris-HCl, pH 6.8 containing 0.8mM sodium pyrophosphate, 5mM EDTA, 2% w/v SDS, 10% glycerol, 001% w/v bromophenol blue, 50mM DTT) (Laemmli, 1970) was then added. A cell scraper was used

to harvest the cells and the cell lysate homogenized through a fine 23G needle several times. Samples were boiled for 5min and then stored at -20°C until analysis.

3.1.2.1.2. Determination of protein concentration

Protein assay was applied on cell lysate (without bromophenol blue) using BioRad protein assay kit (500-0120). The protocol supplied by the manufacturer was followed. Serial dilutions of BSA in PBS (0.2mg/ml – 1.5mg/ml) were prepared and used as standards. 5µl of all of the standards and the cell lysate were added to a 96 well plate in triplicate. 25µl reagent A^* (2% v/v reagent S in reagent A) was added to each well and then 200µl reagent B was added to each well. The plate was incubated for 15min at room temperature with gentle agitation. Within an hour, absorbance was read at 750nm. The volume of sample containing 30µg of protein was then calculated and used in the following steps.

3.1.2.1.3. Resolution of protein by SDS PAGE

A Biorad mini protean gel electrophoresis kit was used to run all gels. Tris buffers were prepared and stored at room temperature for both resolving gel (1.5M Tris and 0.4% w/v SDS, pH 8.4) and stacking gel (0.5M Tris and 0.4 w/v SDS, pH 6.8). 10% resolving gel (appendix 2) was prepared and poured in-between glass plates and spacers (1.5mm thick) to a level 1cm below the bottom of the combs when inserted and overlaid with 1% w/v SDS solution. Once the resolving gel polymerized, a 3% stacking gel (appendix 2) was poured on the top and a 1mm comb was inserted. Following gel polymerization, they were connected to an electrode and placed in a Perspex tank containing electrophoresis buffer (25mM Tris base, 192mM glycine and 0.1% w/v SDS, pH 8.3). Appropriate volume containing 30µg were loaded per well and also 5µl of control pre-stained protein ladders

and of biotinylated standards were uploaded into two of the wells. Samples were electrophoresed at a constant voltage of 100V for 2h.

3.1.2.1.4. Western Blotting

A Biorad mini transfer kit was used for the transfer of proteins to PVDF membrane. PVDF membranes were pre-wet in methanol for 5min and then washed with distilled water and blotting buffer (25mM Tris base, 192mM glycine and 20% v/v methanol) for 15 min each. A transfer sandwich was prepared containing sponge, blotter paper, resolving gel and the pre-wet PVDF membrane. This sandwich was inserted into a transfer tank filled with blotting buffer. The whole tank was engulfed with ice and connected to the power supply at 100V for 2h.

3.1.2.1.5. Detection of protein

Once transfer was completed, PVDF membrane was placed in blocking solution of 10% w/v BSA in tris buffer saline-Tween (TBS-T) (150mM sodium chloride, 20mM Tris base pH 7.4) containing detergent (Tween 20) at a concentration of 0.1% v/v. The membrane was then incubated with phospho-SAPK/JNK mouse mAb (1:2000) or phospho-SAPK/JNK rabbit pAb (1:5000) overnight with agitation at RT. The following day, the membrane was washed 4x for 15min with 20ml TBS-T and then incubated with either horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (1:10000) or horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG (1:10000) for 1h at room temperature depending on which primary Ab was used. Membrane was washed as previously described.

3.1.2.1.6. Detection of protein by enhanced chemiluminescence (ECL)

Following the final wash, membrane was incubated in equal amount of ECL solution 1 and 2 for 90s with gentle agitation and then placed in developing cassette, covered with saran wrap excluding air bubbles. Amersham X-ray film was exposed to the membrane in the dark for up to 10min. Film was immersed in developing solution until bands were visible, then it was washed with tap water before immersing it in fixing solution until the film became translucent. Film was rinsed thoroughly with tap water and left to air dry.

All western blotting experiments were performed on at least 3 independent samples and densitometric analysis of ECL exposure was performed using ImageJ software.

3.1.2.2. JNK activity assay

Ready kit (catalog # 9810) from Cell Signaling Technology Inc, Danvers, MA, USA was used:

3.1.2.2.1. Antibodies

Rabbit polyclonal IgG phospho-c-Jun (Ser63) antibody was obtained from Cell Signaling Technology Inc, Danvers, MA, USA (unique to the kit #9810) and used after diluting (1:10000). ECL anti rabbit IgG horseradish peroxidase linked whole antibody (NA934V) was obtained from GE healthcare UK limited, Buckinghamshire, UK and used after diluting (1:5000). The diluent which has been used to dilute all antibodies was TBS-T including 2% w/v BSA.

3.1.2.2.2. Preparation of cell lysate for JNK activity assay

Following the appropriate stimulation, cells were harvested under nondenaturing conditions. The reaction was stopped by placing the plates on ice and rinsing twice with ice-cold PBS. 500µl of ice-cold cell lysis buffer (20 mM Tris (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM b-glycerolphosphate, 1mM Na_3VO_4, 1µg/ml leupeptin) was then added. A cell scraper was used to harvest the cells and the cell lysate was sonicated on ice for 5s, 3 times. The cell lysates were then microcentrifuged at 14000*g* for 10min at 4°C. The supernatant (cell lysate) was collected and stored at -80°C until analysis.

3.1.2.2.3. Immunoprecipitation with immobilized c-Jun fusion protein

20µl of immobilized c-Jun fusion protein beads slurry was added to 200µl cell lyasate and incubated with gentle rocking overnight at 4°C.

3.1.2.2.4. Kinase assay

Cell lysate/immobilized c-Jun fusion protein was microcentrifuged at 14000*g* for 30s at 4°C. The pellet then was washed twice with 500µl cell lysis buffer with microcentrifuging after each wash (14000*g* for 30s at 4°C). The washing steps were repeated using 500µl Kinase buffer (25mM Tris (pH 7.5), 5mM b-glycerolphosphate, 2mM DTT, 0.1mM Na_3VO_4, and 10mM $MgCl_2$). Samples were kept on ice between and during washes. Following washing steps, the pellet was suspended in 50µl kinase buffer supplemented with 200µM ATP and incubated for 30min at 30°C. The reaction was then terminated by adding 25µl 3x SDS sample buffer (187.5mM Tris-HCl (pH6.8 at 25°C), 6% w/v sodium dodecyl sulfate (SDS), 30% v/v glycerol, 150mM dithiothreitol (DTT), 0.03% w/v bromophenol blue). Samples then were vortexed and microcentrifuged for 30s.

3.1.2.2.5. Western immunoblotting

Samples were heated to 95-100°C for 2-5min then loaded on 10% SDS-PAGE gel and electrophoresis and western blotting carried out as detailed in section 3.1.2.1.

Other techniques which have been used in this chapter to detect the HUVEC:neutrophil interactions and CAM expression have been previously described in section 2.1.

3.2. RESULTS

3.2.1. The effects of chronic exposure to DL-Hcy on the activation of JNK

HUVEC were cultured in media containing 1mM of DL-Hcy for a period of 5-9 days, including at least one passage. Cells were also stimulated with TNF-α (120U/ml; 20min) as a positive control. Antibodies against phosphorylated JNK were used to detect JNK activation in the cell lysates. Results from repeated protein assays showed the protein concentration in the cell lysates from confluent 35mm dishes was reproducibly just below 1mg/ml (Figure 26).

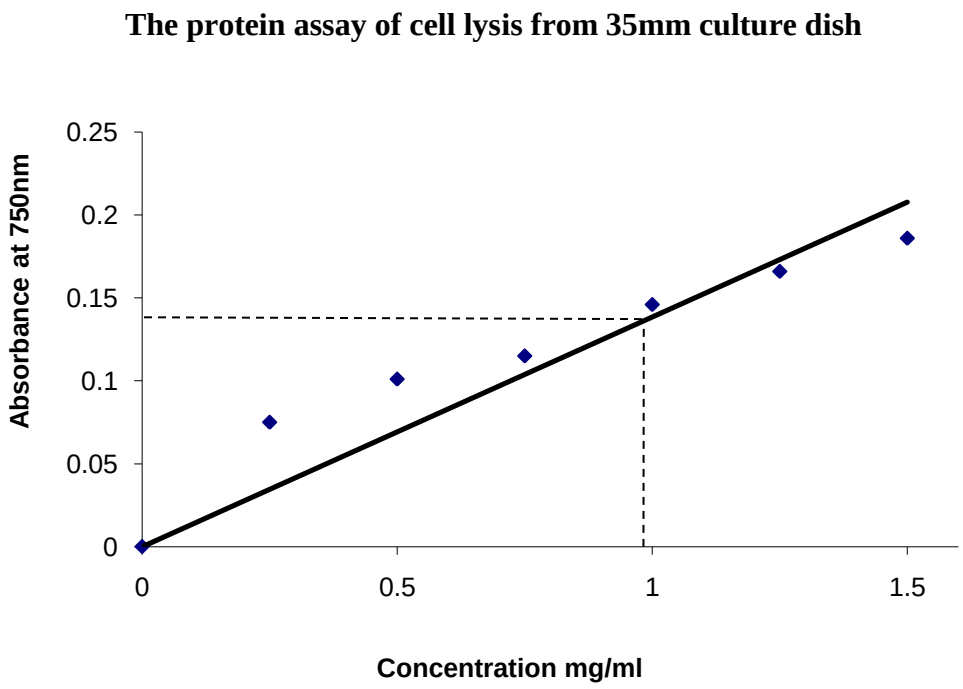


Figure 26: Representative graph showing the results of one protein assay. A serial dilution of BSA in PBS was prepared and absorbance was read at 750nm. The absorbance of this sample was 0.14 and when it is plotted on the graph, protein concentration showed to be nearly 1mg/ml.

Western blotting was conducted to verify if treatment with DL-Hcy resulted in detectable phosphorylated JNK protein. Figure 27A shows protein bands of 46kD and 54kDa representing phosphorylated JNK1 and JNK2 respectively for both DL-Hcy- and TNF-α-treated cells. Figure 27A is representative of 4 different experiments with samples from 4 different donors. The band densities were quantified using ImageJ and the results are shown as the mean number from 4 different experiments +/- SD (Figures 27B and 27C for JNK1 and JNK2 respectively). HUVEC grown in complete M199 media showed less intense bands suggesting that JNK was not phosphorylated in non-treated cells. However, HUVEC grown in media containing DL-Hcy (1mM), showed significantly activated JNK1 presented by the bands of p-JNK1 (46kDa) ($p<0.05$) (Figure 27A) and quantified in Figure 27B. DL-Hcy-treated HUVEC also showed some increase in phosphorylation of JNK2 (54kDa) compared to non-treated cells (Figure 27A) however, this increase was insignificant ($p>0.05$) indicating that chronic treatment with DL-Hcy did not significantly activate the JNK2 isoform (Figure 27C). Furthermore, Figure 27A shows that TNF-α induced activation of both isoforms, JNK1 and JNK2.

The effects of chronic exposure to DL-Hcy on the activation of JNK isoforms

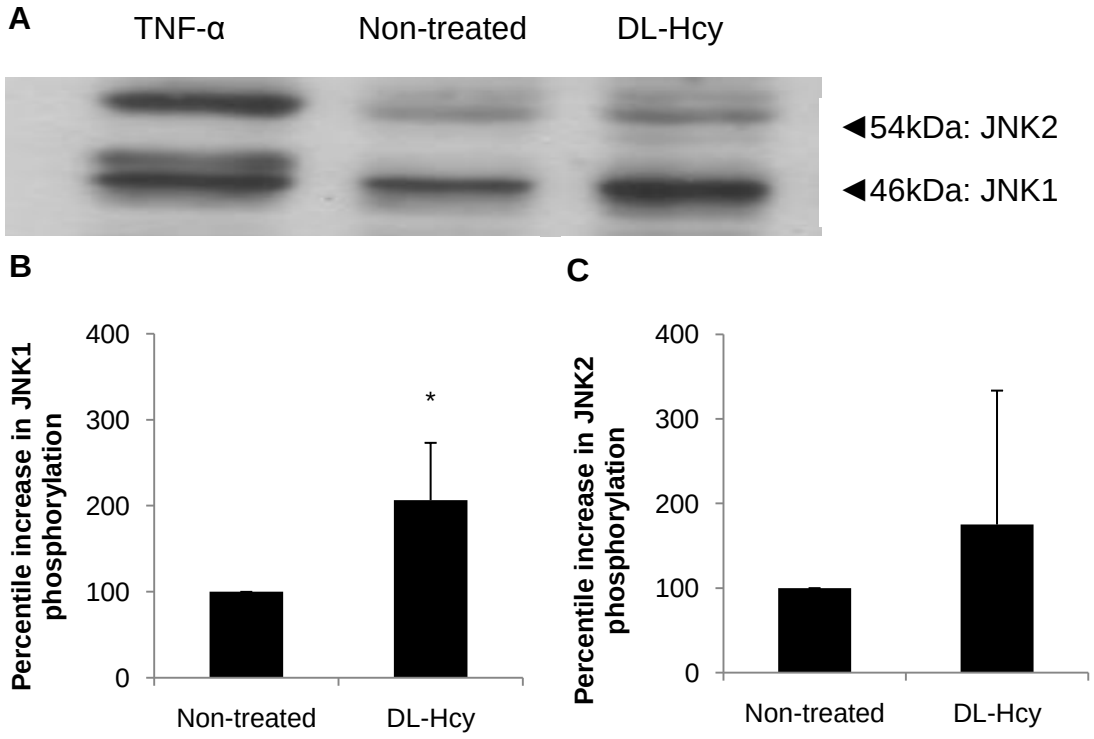


Figure 27: A: Representative blot showing the effects of chronic exposure to DL-Hcy on the activation of different JNK isoforms in HUVEC. HUVEC were cultured in media containing DL-Hcy (1mM) for 5-9 days until confluence and then lysed. TNF-α (120U/ml, 20min) was used as a positive control. 30µg protein from each sample was used in the western blot. Phosphorylated JNK was detected using specific antibodies and was identified as a band of approximately 46kDa (JNK1) and 54kD (JNK2). B and C: Density of both 46kDa (JNK1) and 54kDa (JNK2) bands represented in Figure 27A and from 3 further experiments with independent samples were quantified using ImageJ software. These graphs represent the results for non-treated and DL-Hcy-treated HUVEC which are expressed as mean (+/- SD) from 4 different donors for DL-Hcy and non-treated (n=4). Non-treated values were defined as 100% and DL-Hcy values were adjusted accordingly. (*) $p < 0.05$ for DL-Hcy versus non-treated cells.

3.2.2. The effects of chronic exposure to DL-Hcy and SP600125 on the activation of c-Jun

The activation of JNK in response to stimulation with DL-Hcy alone or in combination with SP600125 was assessed by measuring the activation of the downstream substrate of JNK, c-Jun. Figure 28A shows a protein band of 48kD representing phosphorylated c-Jun for both DL-Hcy- and TNF-α-treated cells (positive control). Figure 28A is representative of 3 different experiments with 3 different donors. The band densities were quantified, and are shown as the mean number from 3 different experiments +/- SD (Figure 28B). Cells which were grown in media containing 1mM DL-Hcy alone showed significantly activated c-Jun represented by bands of phosphorylated c-Jun (approximately 5-fold increase) ($p<0.05$). However, cells which were treated with DL-Hcy (1mM) along with SP600125 (3μM) did not show activated c-Jun. Cells from the same donors were treated with 1mM Cys and those showed no activation of JNK. SP600125 alone was not able to induce any activation of c-Jun.

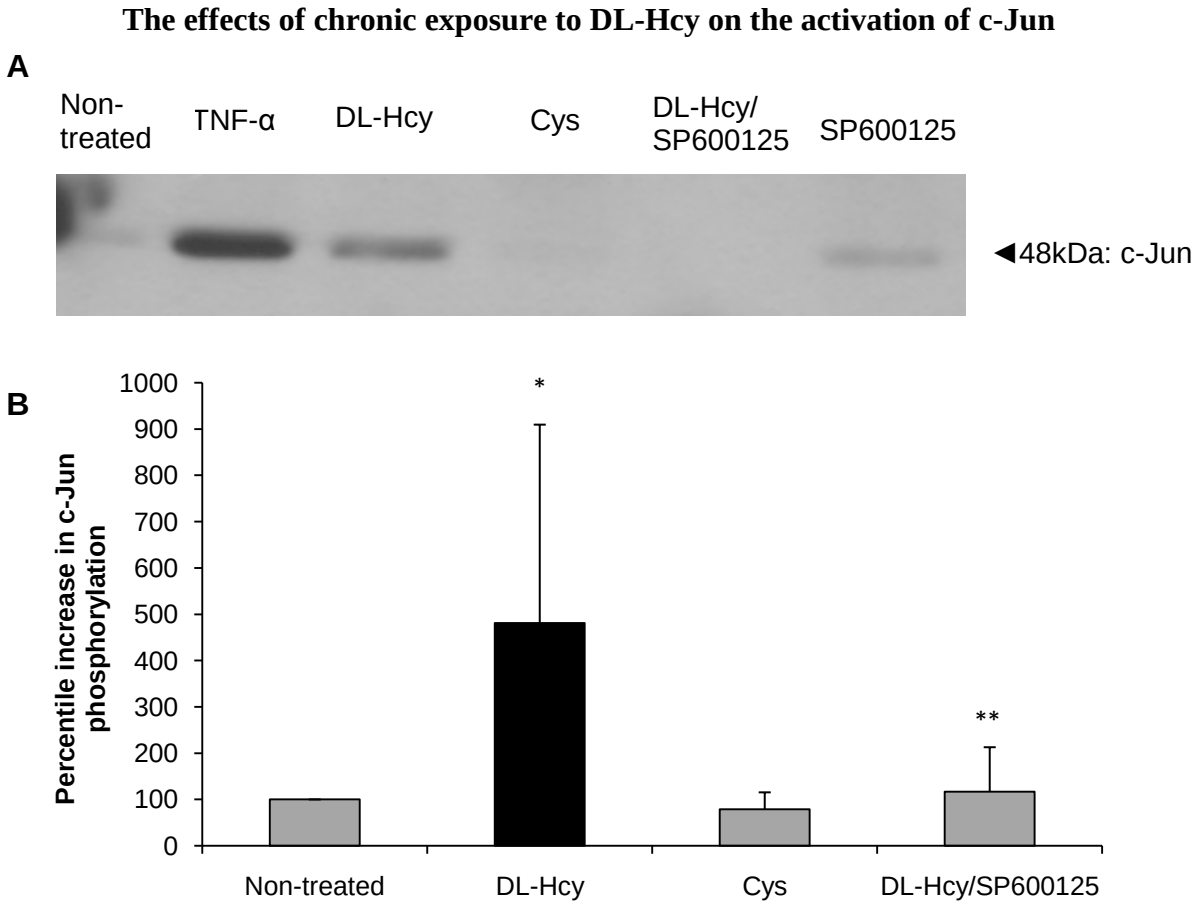


Figure 28: A: Representative blot showing the effects of chronic exposure to DL-Hcy on the activation of c-Jun in HUVEC. HUVEC were cultured in media containing 1mM of DL-Hcy, 1mM DL-Hcy and 3µM SP600125, or 1mM Cys for 5-9 days until confluence and then lysed. TNF-α (120U/ml, 20min) was used as a positive control. Non-treated and Cys-treated HUVEC from the same donor were used in each experiment as negative controls. 30µg protein from each sample was used in western blot. Phosphorylated c-Jun was detected using specific antibodies and was identified as a band of approximately 48kD. B: Density of the bands represented in Figure 28A and from 2 further experiments with independent samples were quantified using ImageJ software. These graphs represent the results for non-treated, DL-Hcy alone or with SP600125, and Cys-treated HUVEC which are expressed as mean (+/- SD) from 3 different experiment (n= 3). Non-treated values were defined as 100% and other values were adjusted accordingly. (*) $p < 0.05$ for DL-Hcy versus non-treated cells, (**) $p < 0.05$ for DL-Hcy versus DL-Hcy/Sp600125-treated cells.

3.2.3. The role of JNK in mediating DL-Hcy-induced CAM expression

Specific FITC-tagged antibodies against E-selectin, ICAM-1 and P-selectin were used to study the expression of these CAM on DL-Hcy-treated HUVEC as described in section 2.1.2.6. Both E-selectin and ICAM-1 showed high fluorescence in response to DL-Hcy treatment (Figures 29B and 30B) compared to non-treated (Figures 29A and 30A). However, such fluorescence was not evident in response to DL-Hcy/ SP600125 for both E-selectin and ICAM-1 (Figures 29C and 30C). Quantifying these results using ImageJ software revealed that DL-Hcy induced significant expression of E-selectin (Figure 29D) and ICAM-1 (Figure 30D) compared to non-treated controls ($p<0.05$). On the other hand, SP600125 prevented the effects of DL-Hcy on E-selectin and ICAM-1 expression to a level similar to non-treated controls ($p>0.05$) and significantly lower than DL-Hcy-treated cells ($p<0.05$) (Figure 29D and 30D).

P-selectin did not show high fluorescence in response to DL-Hcy treatment (Figure 31B) or DL-Hcy/SP600125 treatment (Figure 31C) compared to non-treated controls (Figure 31A). Quantifying these results using ImageJ software revealed that DL-Hcy induced slight increase in P-selectin expression compared to non-treated controls and this increase was prevented by SP600125 treatment (Figure 31D). However, there were no significant differences in the level of P-selectin expression between the three groups ($p>0.05$).

3.2.4. The role of JNK in mediating DL-Hcy effects on EC:neutrophil interactions

Compared with non-treated cells, cells treated with DL-Hcy showed a significant increase in their interactions with flowing neutrophils (as explained in section 2.2.3.6). When HUVEC were treated with DL-Hcy and the JNK/SAPK inhibitor SP600125, the

numbers of tethered, rolled, fixed and transmigrated neutrophils were decreased significantly to a level similar to the numbers of interactions of non-treated cells (Figure 32). Vehicle samples were prepared where cells were treated with SP600125 alone and those cells showed no activation and the numbers of different interactions were similar to those of non-treated cells (data not shown).

The role of JNK in mediating DL-Hcy effects on E-selectin expression

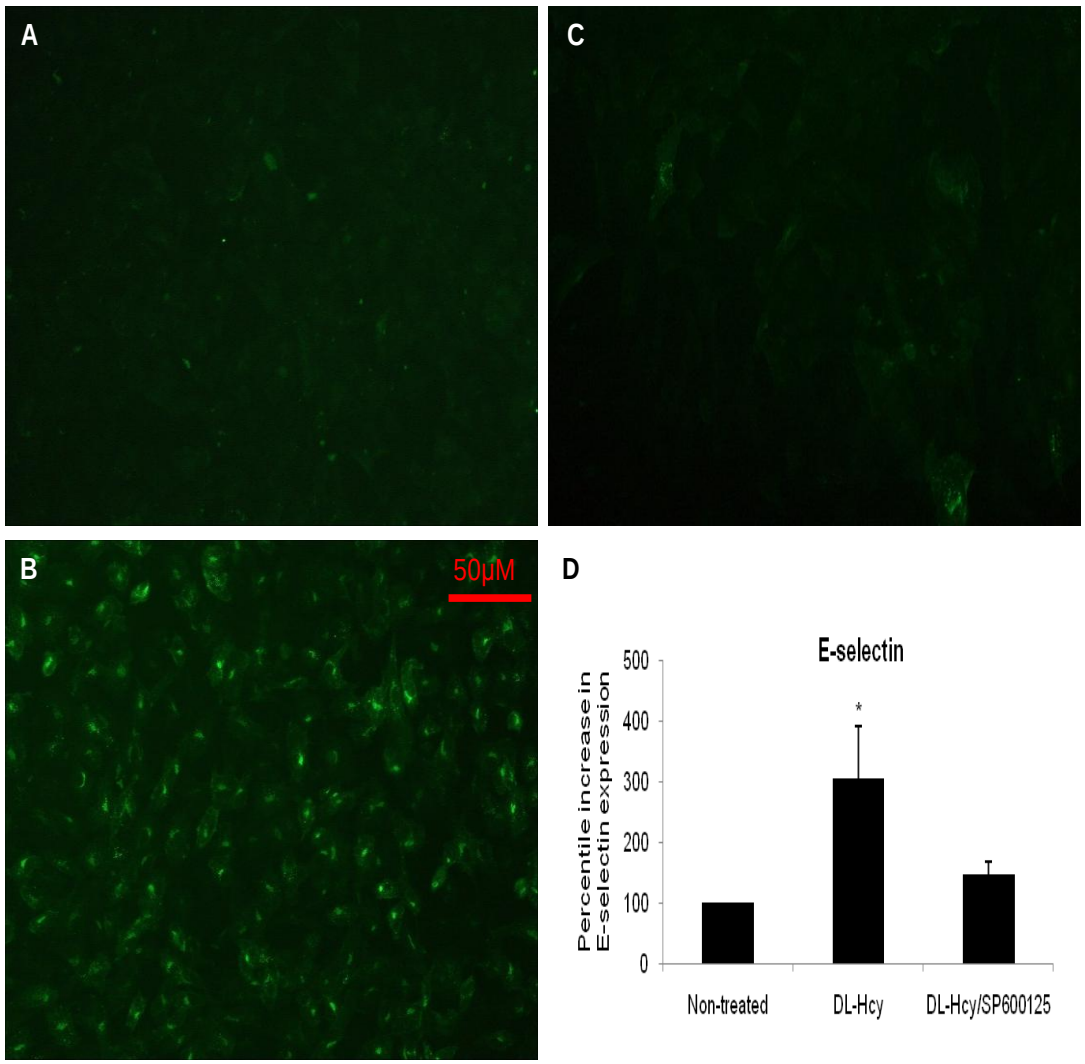


Figure 29: Representative images showing the effect of DL-Hcy on E-selectin expression on HUVEC surface in the presence and absence of SP600125. HUVEC were grown in complete M199 medium and either not treated or treated with DL-Hcy (1mM) alone or with SP600125 (3µM) for 5-9 days. Then, fixed cells were immunostained with FITC-tagged antibodies against E-selectin. A: non-treated HUVEC, B: DL-Hcy-treated HUVEC, C: DL-Hcy and SP600125-treated HUVEC, D: Fluorescence density of the A, B and C was quantified using ImageJ software. These graphs represent the results for DL-Hcy- and DL-Hcy/SP600125-treated HUVEC which are expressed as mean +/- SEM (n = 4). Non-treated values were defined as 100% and other values were adjusted accordingly. (*) $p < 0.05$ for DL-Hcy versus non-treated cells and DL-Hcy/SP600125-treated cells.

The role of JNK in mediating DL-Hcy effects on ICAM-1 expression

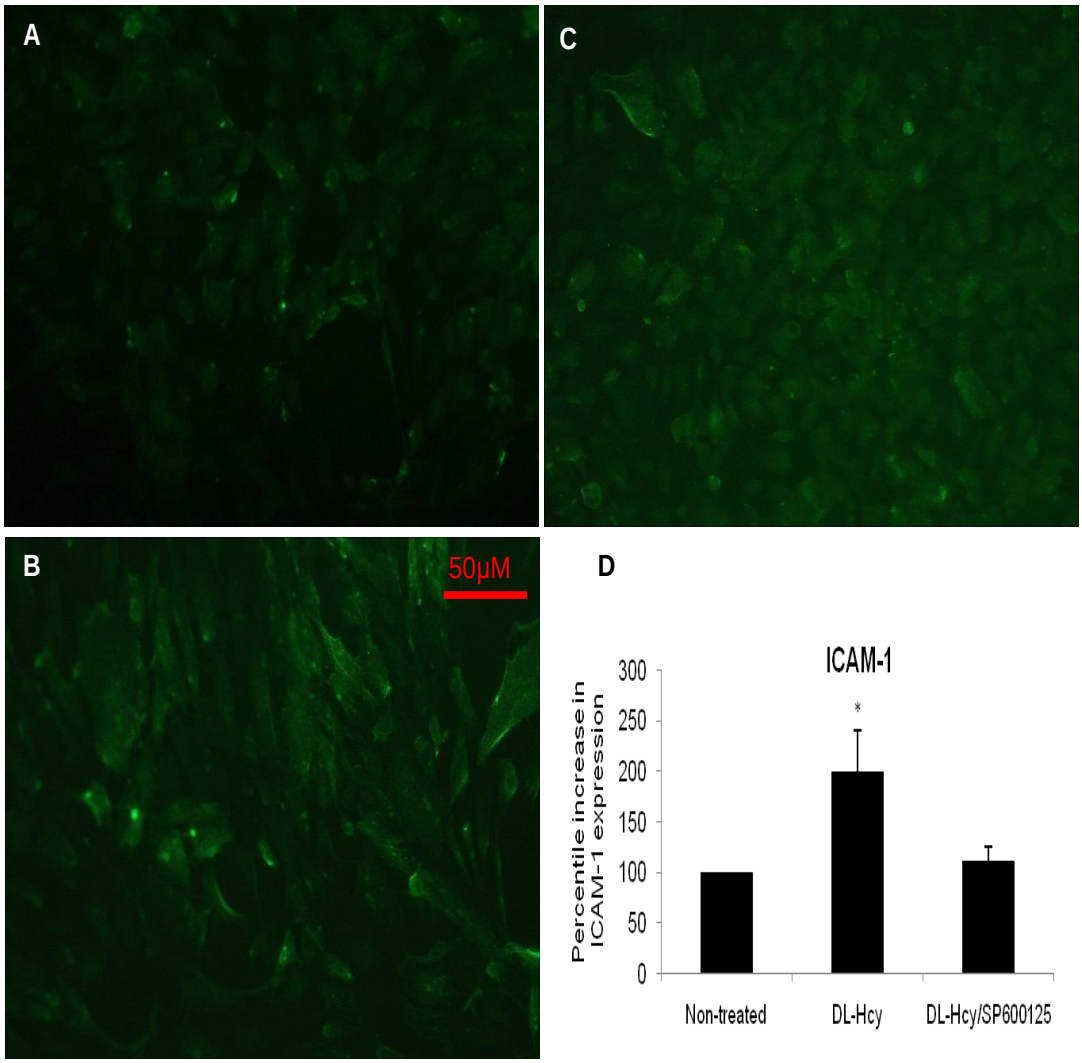


Figure 30: Representative images showing the effect of DL-Hcy on ICAM-1 expression on HUVEC surface in the presence and absence of SP600125. HUVEC were grown in complete M199 medium and either not treated or treated with DL-Hcy (1mM) alone or with SP600125 (3µM) for 5-9 days. Then, fixed cells were immunostained with FITC-tagged antibodies against ICAM-1. A: non-treated HUVEC, B: DL-Hcy-treated HUVEC, C: DL-Hcy and SP600125-treated HUVEC, D: Fluorescence density of the A, B and C was quantified using ImageJ software. These graphs represent the results for DL-Hcy- and DL-Hcy/SP600125-treated HUVEC which are expressed as mean +/- SEM (n = 4). Non-treated values were defined as 100% and other values were adjusted accordingly. (*) $p < 0.05$ for DL-Hcy versus non-treated cells and DL-Hcy/SP600125-treated cells.

The role of JNK in mediating DL-Hcy effects on P-selectin expression

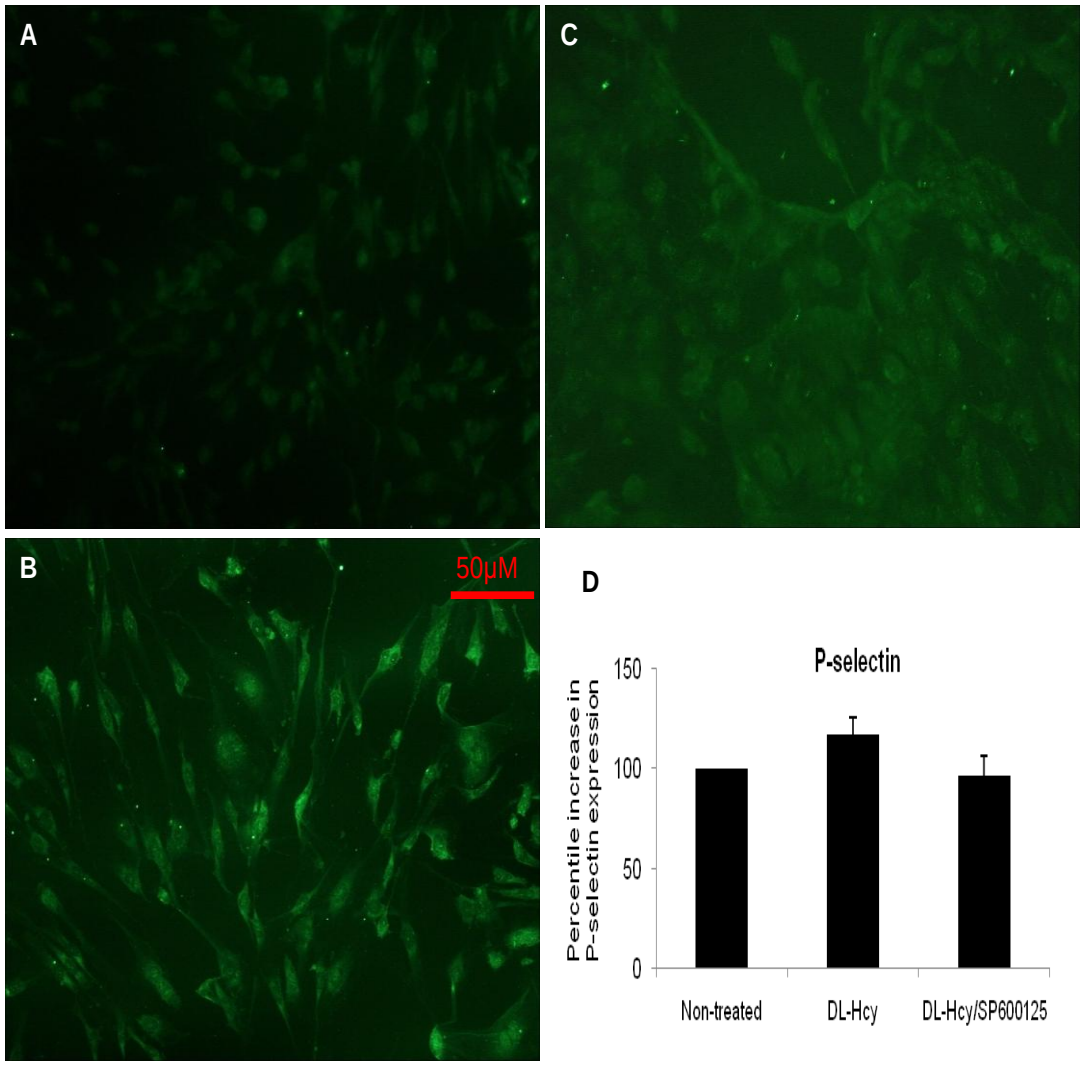


Figure 31: Representative images showing the effect of DL-Hcy on P-selectin expression on HUVEC surface in the presence and absence of SP600125. HUVEC were grown in complete M199 medium and either not treated or treated with DL-Hcy (1mM) alone or with SP600125 (3µM) for 5-9 days. Then, fixed cells were immunostained with FITC-tagged antibodies against P-selectin. A: non-treated HUVEC, B: DL-Hcy-treated HUVEC, C: DL-Hcy and SP600125-treated HUVEC, D: Fluorescence density of the A, B and C was quantified using ImageJ software. These graphs represent the results for DL-Hcy- and DL-Hcy/SP600125-treated HUVEC which are expressed as mean +/- SEM (n = 3). Non-treated values were defined as 100% and other values were adjusted accordingly.

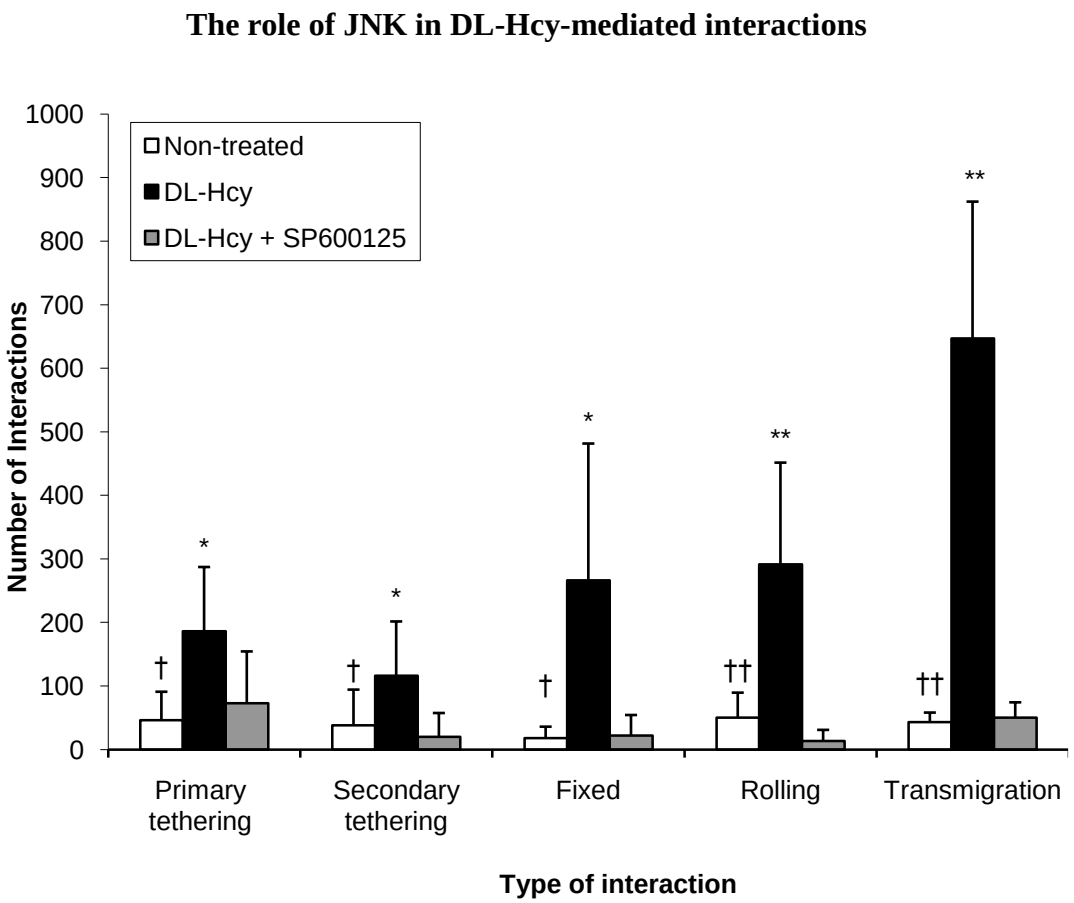


Figure 32: The effects of the JNK inhibitor, SP600125, on HUVEC:neutrophil interactions under flow conditions. HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) alone or with 3µM SP600125 and the effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in media without any stimulation and were considered as negative control (non-treated) in each experiment. The results are expressed as mean (+/- SD) from 5 different donors (n=5). (*) $p < 0.05$, (**) $p < 0.01$ for DL-Hcy versus DL-Hcy/SP600125 treated cells.

3.3. DICUSSION

While the activation of HUVEC by sustained exposure to Hcy has been clearly shown in Chapter 2, the mechanisms responsible require characterisation. JNK is a signal transducer that has been implicated in both cell survival and cell apoptosis (Barr and Bogoyevitch, 2001). The functional cellular outcome downstream of JNK activation depends on the cell type, stimulation type and environmental circumstances (Hoshino *et al.*, 2005). JNK activation is regulated by upstream kinases MKK4/7 and by dual specificity phosphatases that dephosphorylate and inactivate JNK. Therefore, either activation of MKK4/7, decreased JNK phosphatase activity or combination of both can lead to JNK activation (Chen *et al.*, 2001a). Previous studies have shown that JNK is activated in response to acute treatment with Hcy (up to 24h) in HUVEC (Cai *et al.*, 2000; Dong *et al.*, 2005; Zhang *et al.*, 2001b). No studies have been carried out to demonstrate if the activation of JNK is sustained in EC over longer periods of time in response to Hcy. Therefore, in this chapter, HUVEC were exposed to DL-Hcy chronically (5-9 days) and the effects of this extended treatment in HUVEC were investigated to determine whether this treatment induces sustained activation of JNK. DL-Hcy induced a 2-fold increase in the level of phosphorylated JNK1 compared to non-treated control cells ($p<0.05$) on continual stimulation for 5-9 days (Figure 27). It has been suggested that only the large isoform of JNK (54kD) gets phosphorylated in murine atherosclerotic lesions (Ricci *et al.*, 2004). However these authors were not able to specify in which cell type of the lesion the 54kDa JNK isoform was phosphorylated. This chapter's results clearly indicate that only JNK1 (46kDa) was phosphorylated in chronically Hcy-treated HUVEC.

The antibodies used in this chapter recognize the active forms of JNK1, JNK2, and JNK3 isoforms. Therefore, these antibodies were able to recognize both JNK1 (46kDa) and JNK2 (54kDa) which are known to be expressed in EC. The data of this chapter clearly indicate that Hcy preferentially activated the JNK1 isoform which was suggested by Sabapathy *et al* (2004) to play a major role in activating c-Jun. However, TNF-α activated both JNK1 and JNK2 isoforms which means different other substrates will be activated in addition to c-Jun. This will lead to amplifying TNF-α effects compared to Hcy and might explain the robust effects of TNF-α compared to Hcy in inducing EC activation, such as the effects observed in CAM up-regulation in Chapter 2 (Figures 20C and 21C).

Several published acute studies have demonstrated similar findings, however none of them have identified which JNK isoform was activated. Cai's group treated HUVEC with 3mM Hcy which induced significant and rapid phosphorylation of JNK within 30min and this phosphorylation was sustained for the duration of the experiment (8h) (Cai *et al.*, 2000). Zhang's group gave evidence that JNK was still activated after 12h of treatment with 3mM Hcy (Zhang *et al.*, 2001b). Furthermore, Dong *et al* showed that JNK remained activated after 24h treatment with 1mM Hcy (Dong *et al.*, 2005). Therefore, it could be said that JNK stayed activated for the duration of exposure to Hcy (up to 24h). However, 24h treatment was not enough to ensure that cells were exposed to Hcy through all stages of cellular proliferation. Therefore, this chapter confirms the previous findings and additionally has given strong evidence that a longer exposure to DL-Hcy induces continuous activation of JNK after up to 9 days treatment indicating that JNK was always activated in the presence of DL-Hcy (Alkhoury *et al.*, 2009a). This is in contrast to data obtained with other cellular stresses implicated in atherosclerosis such as TNF-α. JNK is

activated in response to TNF-α in HUVEC and peaks after 15min but decreases sharply after 60min with a maximum activation at 30min (Zhou *et al.*, 2007) due to action of JNK phosphatase (Cavigelli *et al.*, 1996). This chapter along with the previously described more acute studies (Cai *et al.*, 2000; Dong *et al.*, 2005; Zhang *et al.*, 2001b) gives evidence for a continuous up-regulation of JNK at all time points measured in response to continuous treatment with Hcy. Therefore, these findings might indicate that chronic exposure to Hcy in patients with hHcy could induce sustained activation of JNK pathway. Despite the clear results in Figure 27, the antibodies used in the western blotting experiments produced variable results from different batches of antibody. Due to the unreliability of the antibodies used from both NEB and Promega, a JNK assay kit was also used to quantify the activation of JNK downstream substrate, c-Jun. In DL-Hcy-treated cells, activated JNK induced approximately a 5-fold increase in the activation of its downstream substrate c-Jun (Figure 28). On the other hand, Cys was not able to induce c-Jun activation which means these effects are not non-specific but are mediated by Hcy.

JNK isoforms have been suggested to interact differently with different substrates such as c-Jun and ATF-2 (Gupta *et al.*, 1996). JNK2 was found to have 10-times and 25-times higher affinity to c-Jun than JNK1 in COS-1 cells (Sluss *et al.*, 1994) and in Jurkat cells (Kallunki *et al.*, 1994) respectively. Both studies suggested that JNK2 preferentially phsphorylates c-Jun due to its high binding affinity compared to JNK1. However, Sabapathy *et al* (2004) suggested that this binding affinity represents a mechanism by which JNK2 works as a negative regulator of c-Jun stability and that, in unstimulated fibroblasts, most c-Jun is found bound to JNK2. Once these cells are stimulated, JNK2 leaves the c-Jun therefore, giving more access to activated JNK1 leading to c-Jun

phosphorylation (Sabapathy *et al.*, 2004). Therefore, Sabapathy's group suggested JNK1 to be the main contributor to c-Jun activation. Furthermore, the same group found JNK1 to preferentially bind and activate ATF2 (Sabapathy *et al.*, 2004). Collectively, it is very clear that different JNK isoforms interact differently with different substrates. DL-Hcy was shown to preferentially activate JNK1 which might preferentially activate c-Jun compared to JNK2. Even though activated JNK1 was able to induce significant activation of the downstream c-Jun in this chapter, this activation was less than that induced by TNF-α and that was clearly because TNF-α was able to induce more activation of JNK1 in addition to activating JNK2.

Cumulatively, this chapter shows that chronic treatment of HUVEC with DL-Hcy activated JNK1 isoform (46kDa) and although it is yet unknown whether the activity of JNK would be affected by which isoform is being phosphorylated, this chapter also demonstrates that DL-Hcy-induced activation of the JNK1 was efficient enough to induce activation of its substrate c-Jun and mediated cellular effects on CAM expression. However, it is not currently clear whether Hcy effects are via activating MKK4/7 and/or decreasing in JNK phosphatase activity.

It was demonstrated in Chapter 2 that DL-Hcy induced P-selectin, E-selectin and ICAM-1 expression which in turn mediates different sorts of HUVEC:neutrophil interactions. However, a little is known about the pathway through which Hcy induces CAM up-regulation. Therefore, to study to which extent JNK is implicated in mediating Hcy effects on CAM expression, the JNK specific inhibitor SP600125 was used and cells were incubated with SP600125 and DL-Hcy together for 5-9 days. SP600125 has been previously shown to selectively inhibit JNK with an IC50 of 40nM and to have no

significant effects on other MAP kinase activities (Bennett *et al.*, 2001; Bogoyevitch, 2005). SP600125 has been used as a JNK specific inhibitor by several *in vivo* studies reviewed by Sumara *et al* (2005) and *in vitro* studies (Ennis *et al.*, 2005; Miho *et al.*, 2005; Zhou *et al.*, 2007). SP600125 showed a higher selectivity for the JNK pathway over other inflammatory response mediators in Jurkat T cells (Bennett *et al.*, 2001). 3µM SP600125 was used in this chapter and gave a strong inhibition of JNK activation. The results of the JNK activity assay in Figure 28 show that DL-Hcy-mediated c-Jun activation was totally reversed by SP600125 thus showing effective blockade of DL-Hcy-induced JNK pathway activation. Thereafter, the role of JNK in mediating DL-Hcy induced CAM expression was studied under both static and flow conditions. Using FITC-tagged antibodies against P-selectin, E-selectin and ICAM-1 as explained in Chapter 2 it was revealed that SP600125 suppressed DL-Hcy-induced E-selectin and ICAM-1 expression (Figures 29 and 30). Quantifying the images from 4 independent experiments on cells from different donors using ImageJ showed that there was no significant difference in expression between DL-Hcy/SP600125-treated cells and non-treated cells ($P>0.05$), while significant difference in expression was observed between DL-Hcy-treated cells and DL-Hcy/SP600125-treated cells ($p<0.05$) (Figure 29D and 30D). On the other hand, P-selectin expression showed no significant differences between all types of treatment and non-treated cells (Figure 31). Therefore, blocking the JNK pathway inhibited DL-Hcy-induced E-selectin and ICAM-1 expression while results of P-selectin were not interpretable as the immunofluorescence technique was not able to reveal significant P-selectin expression in response to Hcy alone (as explained in Chapter 2). Further experiments to determine the functional significance of the alterations in adhesion molecule expression were carried out under flow conditions

where neutrophil recruitment was quantified. HUVEC treated with DL-Hcy and SP600125 were subjected to flowing neutrophils and interactions were counted. DL-Hcy alone induced significant increases in all types of interactions between HUVEC and neutrophils compared to non treated controls ($p<0.05$ in all cases) (as explained in Chapter 2). However, blocking JNK pathway activation by SP600125 was able to reverse all of these effects. Numbers of tethered, rolled, adhered and transmigrated neutrophils dramatically decreased and were similar to non-treated cells (p values were ranging from 0.2 to 0.8) (Figure 32). The dramatic decrease in the number of rolling and adhered neutrophils is likely to reflect a down-regulation of the expression of E-selectin and ICAM-1 respectively which are known to mediate these interactions. Therefore, it could be said that JNK plays a key role in mediating DL-Hcy effects on E-selectin and ICAM-1 expression on the HUVEC surface. The immunofluorescence results confirm that JNK is mediating the up-regulation of E selectin and ICAM-1 on the EC surface in response to DL-Hcy. These findings match with other studies which showed that JNK mediates TNF-α-induced E-selectin up-regulation in HUVEC (Min and Pober, 1997). The results in this chapter are also in agreement with the findings of Miho's group, who showed that thrombin-induced ICAM-1 up-regulation is mediated by JNK in both HUVEC and BAEC by a mechanism that is dependent on G proteins (Miho *et al.*, 2005). However, different mechanisms by which Hcy induces JNK activation were suggested like induction of oxidative stress (to be discussed in Chapter 4). Miho's group used the same JNK inhibitor which has been used in this chapter SP600125 but at a higher concentration (10 and 30μM) and it showed inhibition of thrombin up-regulation of ICAM-1 in HUVEC. However, once they used equivalent concentrations of SP600125 to those used in this chapter, there was no

significant inhibition of thrombin-induced ICAM-1 up-regulation. It might be that thrombin induced robust activation of JNK compared to that induced by Hcy in this chapter. Therefore, higher concentrations of the JNK inhibitor SP600125 were needed to overcome this thrombin-induced JNK robust activation than those used to overcome Hcy-induced JNK activation.

Zhou's group found that 30μM SP600125 significantly inhibited TNF-α-induced JNK activation in HUVEC by 75% (Zhou *et al.*, 2007). However, they found that JNK was not involved in TNF-α-induced ICAM-1 up-regulation as 30μM SP600125 was not able to significantly reverse TNF-α effects and they suggested it to be NF-κB-mediated instead. SP600125 was recommended to be specific for JNK inhibition at concentration of 3μM which was enough to totally inhibit the JNK pathway activation as this chapter shows. Furthermore, this concentration of SP600125 totally reversed Hcy-induced ICAM-1 up-regulation under static and flow conditions. Collectively, that suggests a strong involvement of JNK in ICAM-1 Hcy-mediated expression; Zhou's study however suggests that TNF-α-mediated ICAM-1 expression happens mainly through NF-κB-mediated pathway. These data together suggest that ICAM-1 expression could be mediated by different pathways depending on the stimuli.

While immunofluorescence studies on P-selectin could not confirm up-regulation of this adhesion molecule, the flow data strongly suggests that some up-regulation is going on and this is confirmed by the tethering reactions in response to Hcy under flow. The inhibition of tethering when the JNK pathway is blocked strongly implicates a role for JNK in P-selectin expression, a previously unreported phenomenon. P-selectin is presynthesized and stored in WPB in the EC and will be mobilized to the cell surface once appropriate

stimuli are present (Hattori *et al.*, 1989). Chronic exposure to certain appropriate stimuli, like TNF-α, induces continuous expression of P-selectin on mouse EC surface (Hahne *et al.*, 1993). Half of the expressed P-selectin will be shed by proteolysis (Hartwell *et al.*, 1998) and the other half internalised and recycled to WPB (Arribas and Cutler, 2000). Therefore, there must be a synthesizing mechanism for new P-selectin to guarantee its continuous expression. TNF-α was shown previously to induce P-selectin in mice in a mechanism similar to TNF-α-induced E-selectin synthesis (Hahne *et al.*, 1993). JNK in this chapter was shown to play a role in DL-Hcy-mediated P-selectin expression measured by number of tethered neutrophils under flow conditions; however, P-selectin expression could result from WPB exocytosis or from *de novo* synthesis. The flow experiments from the first chapter provided evidence that DL-Hcy induced continuous expression of P-selectin on HUVEC and this chapter provided evidence that JNK activation is required for this expression. These results together indicate for the first time that JNK is involved in mediating synthesis of P-selectin and/or WPB exocytosis in HUVEC. Therefore, the question which remains unresolved is whether JNK mediates WPB exocytosis or *de novo* synthesis of P-selectin.

The use of MAP kinase inhibitors like SP600125 has been criticized in several reports reviewed by Bain *et al* (2003). It is argued that it might not be very specific and might interfere with other proteins which might give misleading results. In fact, another technique may be used to confirm the findings of this chapter, small interfering (siRNA) could be used to down-regulate the expression of JNK in HUVEC and then study whether P-selectin synthesis and/or expression is affected. On the other hand, the mechanism by which Hcy is inducing JNK pathway activation is still unknown and the next chapter will

focus on the ability of Hcy to induce ROS generation and whether those radicals are involved in mediating the JNK pathway activation.

3.4. CONCLUSION

The JNK MAP kinase pathway is a key pathway in DL-Hcy-mediated HUVEC activation and potential damage. JNK mediates DL-Hcy-induced CAM up-regulation which in turn mediates the HUVEC:neutrophil interactions under flow conditions. Blocking this pathway with a specific JNK inhibitor (SP600125) reversed all the effects of DL-Hcy and provided an evidence of potential therapeutic benefit of JNK specific inhibitors in preventing unwanted inflammatory responses.

CHAPTER 4

4. THE ROLE OF OXIDATIVE STRESS IN HCY-MEDIATED EC ACTIVATION

Oxidative stress is a cellular condition which results from high levels of ROS and/or impaired defective function of antioxidant defence systems (Sies, 1997) and it has been shown in many studies to be associated with cardiovascular diseases (Cai and Harrison, 2000; Dimmeler *et al.*, 1997; Kanani *et al.*, 1999; Loscalzo, 1996; Sorescu *et al.*, 2002).

Hcy contribution to the onset of cardiovascular disease has more recently been explained by several pathogenic mechanisms including generation of reactive oxygen species (ROS)(Loscalzo, 1996), endoplasmic reticulum stress (Ji and Kaplowitz, 2004), genetic disruption (Kokame *et al.*, 1996) and abnormal fibrinolysis (Hajjar *et al.*, 1998). The majority of evidence from previous reports indicates that oxidative stress could be responsible for Hcy atherogenic effects (Papatheodorou and Weiss, 2007). Several possible theories have been suggested for Hcy-induced oxidative stress including auto-oxidation of the free thiol group in Hcy (Misra, 1974), inhibition of different enzymatic antioxidant systems such as glutathione peroxidase (Handy *et al.*, 2005), the activation of NADPH oxidases (Dong *et al.*, 2005) in addition to decreasing NO bioavailability through direct disruption of eNOS (Zhang *et al.*, 2000) and/or inducing eNOS uncoupling (Heydrick *et al.*, 2004).

From the results of chapters 2 and 3, it is clear that Hcy induces EC activation via a JNK mediated pathway. However, the mechanism by which chronic Hcy induces such

activation is still unclear. Therefore, this chapter investigates the role of oxidative stress in inducing activation of Hcy-treated HUVEC by measurement of oxidant stress and assessment of intracellular signalling pathways involved as well as examining the effects of anti-oxidant treatment on leukocyte recruitment by endothelial cells under flow conditions.

4.1. MATERIALS and METHODS

4.1.1. Materials

N-acetyl cysteine (NAC) (A7250), Vitamin C (A4544), pyridine (184527) and 2′,7′-Dichlorofluorescin diacetate (DCHF-DA) (D6883) were purchased from Sigma-Aldrich Chemical Company (Poole Dorset, UK). 2′,7′-Dichlorofluorescin diacetate (DCHF-DA) (287810) and nitrotetrazolium blue (NBT) (484235) were supplied by Calbiochem (La Jolla, USA). Phenol red-free M199 media (11043-023) was supplied by Invitrogen (Paisley, UK).

4.1.2. Treatment with antioxidants

HUVEC were cultured for a period of 5-9 days in complete M199 medium containing 1mM DL-Hcy alone or with either 5mM NAC or 100µM vitamin C. HUVEC from same cell line were cultured for a period of 5-9 days in complete M199 medium alone or containing 1mM Cys and considered as negative controls.

4.1.3. Measurement of oxidative stress

4.1.3.1. Measurement of intracellular superoxide anion

NBT reduction was used to measure the intracellular production of superoxide anions. The protocol recommended by Eligini *et al* (2005) was used with slight modification after personal communications with Susanna Colli, University of Milan, Italy. Treated HUVEC were seeded into gelatin coated 35mm cell culture dishes (BD Falcon, cat No. 351008) and grown until confluence. Media was aspirated and replaced with Phenol red-free M199 media containing the same treatment for each well in addition to 1mg/ml NBT and cells were incubated at 37°C and 5% CO_2 for 90min. Subsequently,

media was discarded and cells were washed twice with DPBS. Cells were harvested in 1ml PBS by gentle scraping using a sterile scraper. The cell pellet was collected by centrifuging at 2300*g* for 5min. Pellets were lysed vigorously in 500µl 100% pyridine and then cell lysis vortexed thoroughly before heating in a heating block at 95°C for 20min. Absorbance was measured immediately using a spectrophotometer at a wavelength of 570nm after the machine was blanked using 100% pyridine alone. Intracellular superoxide anion production was proportional to the absorbance values (Au-Yeung *et al.*, 2004; Eligini *et al.*, 2005). Concentration of super oxide anions was measured using the absorbance values and the extinction coefficient: 7200 $M^{-1}xcm^{-1}$. All samples were prepared and analysed in duplicate.

4.1.3.2. Detection of total ROS

Treated HUVEC were seeded into gelatin coated chamber slides (Nunc, Roskilde Denmark) and grown until confluence. Media was aspirated and cells were washed twice with DPBS. Media containing 100µM DCFH-DA was added and cells were incubated at 37°C and 5% CO_2 for 45min. DCFH-DA is membrane-permeable and once it gets into the cell, it is converted into non-fluorescent DCFH through the action of cellular esterases. However, in the presence of ROS, DCFH is oxidized to its fluorescent derivative 2', 7'-dichlorofluorescin (DCF). Fluorescence intensity of DCF was read at 525nm emission when exited at 488nm by confocal microscope (Leica, TCS SP-2, Heidelberg, Germany).

4.2. RESULTS

4.2.1. Measurement of intracellular superoxide anion

To investigate whether DL-Hcy induced generation of superoxide anions intracellularly, the NBT reduction assay was used. NBT is water soluble and will be taken up intracellularly where it will be converted in the cytoplasm by the action of superoxide anions to a water insoluble blue formazan crystal (Baehner *et al.*, 1976). The formed crystals are trapped within the cell but can be released by solubilising in solvent solution e.g. pyridine and then formation can be monitored spectrophotometrically at 550-560nm (Tarpey and Fridovich, 2001). Figure 33 shows clearly that there are detectable levels of superoxide anions in non-treated cells. Cys-treated cells also showed detectable levels of superoxide anion but they were not significantly different compared to non-treated cells ($p<0.05$), while cells treated with 1mM DL-Hcy for 5-9 days showed significant increases in superoxide anions compared to non-treated cells (1.5-fold, $p<0.01$).

The effects of chronic exposure to DL-Hcy on superoxide anion production

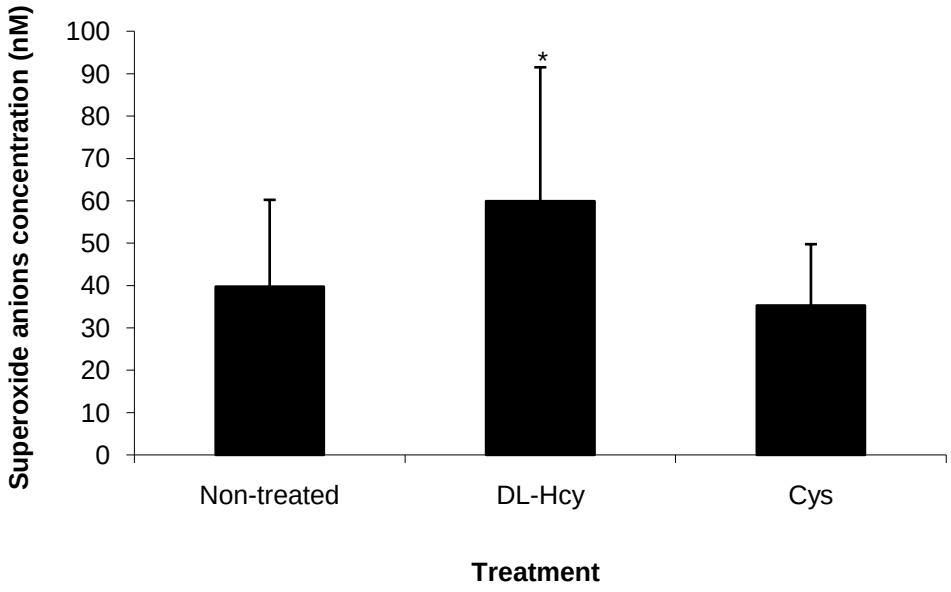


Figure 33: HUVEC were grown in complete medium and either not treated or treated with DL-Hcy (1mM) or Cys (1mM) for 5-9 days. Cys-treated and non-treated cells were considered as negative controls. Media was replaced with phenol read-free media containing NBT (1mg/ml) and incubated at 37°C in 5% CO_2 for 90min. Cells were lysed in pyridine, heated at 90°C for 20min and absorbance was measured immediately at 570nm. Extinction coefficient: 7200 $M^{-1}xcm^{-1}$. The results are expressed as mean (+/- SD) from 10 different donors for DL-Hcy and control (n=10) and 5 different donors for Cys. (*) $p < 0.01$ for DL-Hcy versus non-treated cells.

4.2.2. Detection of total ROS and the effects of NAC on the ROS generation

Different ROS can be produced intracellularly in addition to superoxide anions by EC including peroxides and hydrogen peroxide (Droge, 2002). To investigate whether Hcy might induce increase in the generation of total ROS generated intracellularly the DCFH-DA probe was used. DCFH-DA is a lipid permeable nonfluorescent compound is taken up by cells, where intracellular esterase cleaves the molecule to DCFH which remains trapped inside the cells (Tarpey and Fridovich, 2001). DCFH is oxidized by intracellular ROS to form the lipid-impermeable and fluorescent compound 2', 7' dichlorofluorescin (DCF) (Royall and Ischiropoulos, 1993) (Figure 34).

Even though non-treated and Cys-treated cells produced small amounts of ROS (Figure 35A and 35B), DL-Hcy-treated cells showed a much higher production of the fluorescent dye (Figure 35C) which reflects significant accumulation of ROS. Cells which had been treated with DL-Hcy and NAC together showed very low expression of ROS (Figure 35D) similar to the level of non-treated cells. Data in Figure 35 are representative of two individual experiments using HUVEC from two different donors.

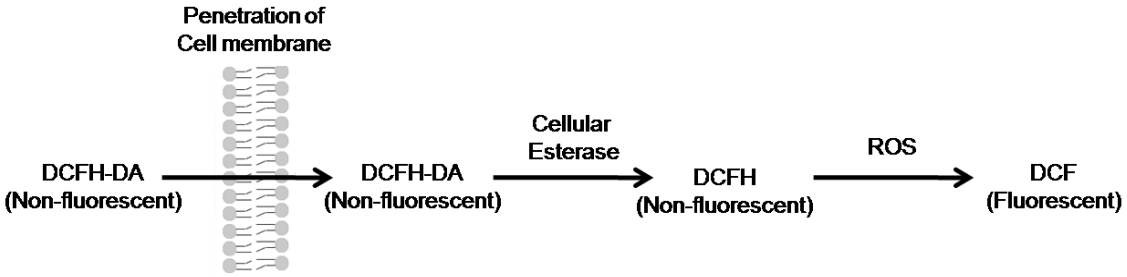


Figure 34: The mechanism of DCFH-DA assay.

The effects of chronic exposure to DL-Hcy and NAC on ROS production

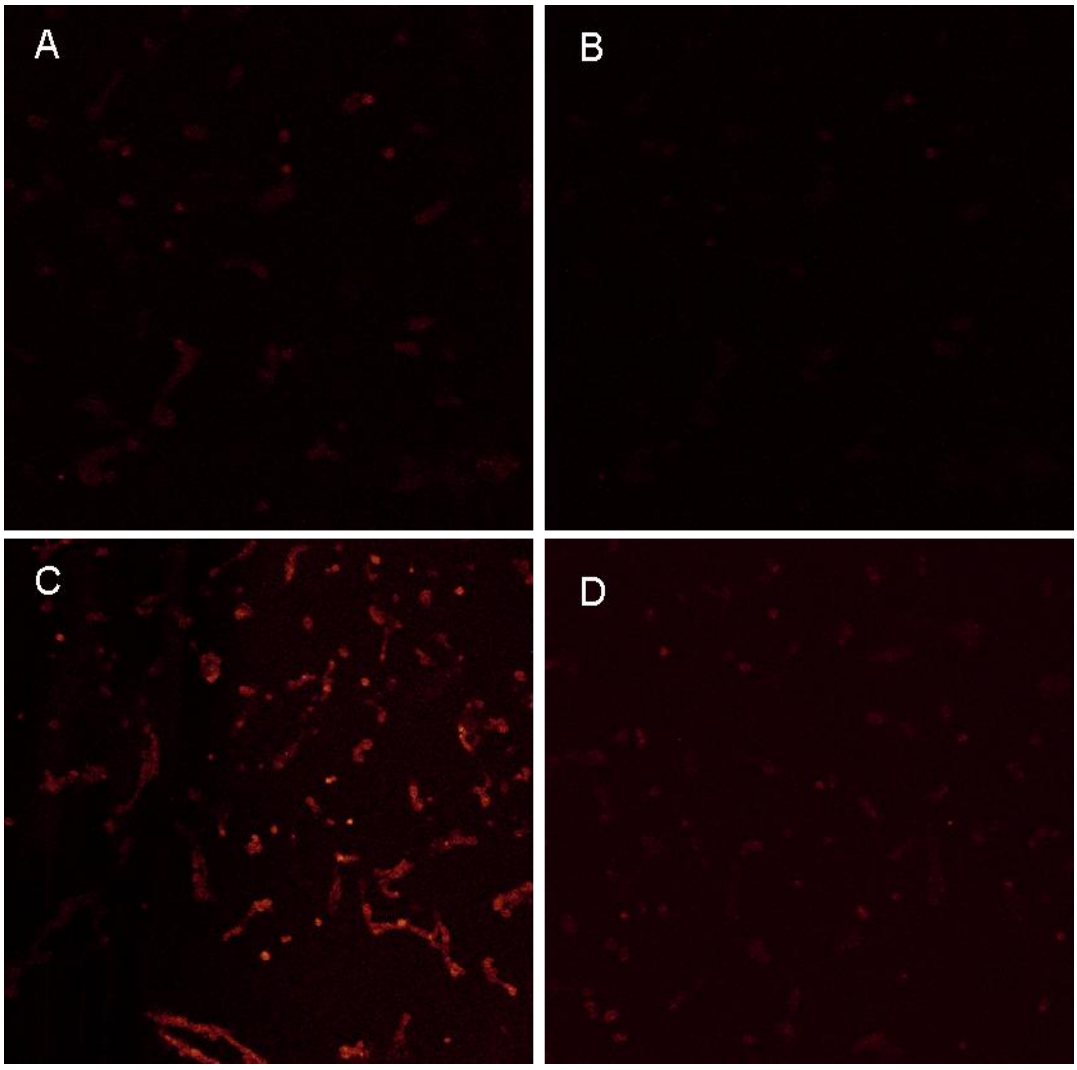


Figure 35: Representative images showing the effect of DL-Hcy and NAC on ROS generation in HUVEC. HUVEC were grown in complete medium and either not treated or treated with DL-Hcy (1mM) alone or in combination with NAC (5mM) for 5-9 days. Cells from the same donor were treated with Cys (1mM) for 5-9 days. Non-treated and Cys-treated cells were considered as negative controls. These cells were washed with DPBS then incubated with 100µM DCFH-DA for 45min. Fluorescence intensity of DCF was read at 525nm emission when exited at 488nm by confocal microscope. A: non-treated, B: Cys, C: DL-Hcy, D: DL-Hcy/NAC.

4.2.3. Oxidative stress and JNK pathway

The results in Chapter 3 demonstrated that chronic treatment with DL-Hcy induced JNK activation. However, it was unclear from these experiments if this activation was dependent on generation of ROS in response to DL-Hcy treatment. Therefore, NAC was used to prevent oxidative stress generation which has been shown in the previous section (section 4.2.2) and thereafter, the JNK activity assay (as described in section 3.1.2.2) was used to study the effects of NAC on DL-Hcy-induced JNK activation and determine if JNK activation was oxidative stress dependent. Figure 36A shows a protein band of 48kD representing phosphorylated c-Jun for both DL-Hcy- and TNF-α-treated cells (positive control). However, no bands were observed for cells which were treated with DL-Hcy and NAC. Figure 36A represent 3 different experiments with 3 different donors. These band densities were quantified as means of 3 different experiments +/- SD (Figure 36B). NAC alone was not able to induce any effects.

The effects of antioxidants on DL-Hcy-induced c-Jun activation

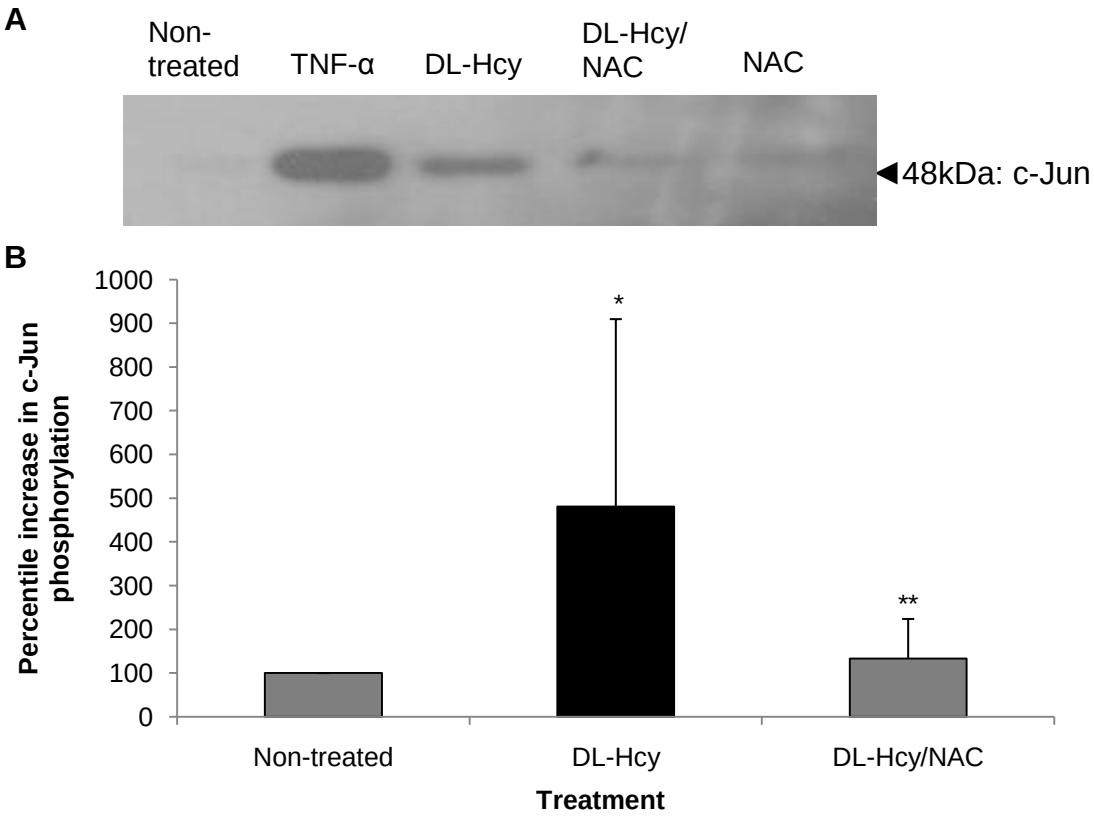


Figure 36: A: Representative blot showing the effects of chronic exposure to DL-Hcy on the activation of c-Jun in HUVEC. HUVEC were cultured in media containing 1mM DL-Hcy, 1mM DL-Hcy and 5mM NAC, or 1mM Cys for 5-9 days until confluence and then lysed. TNF-α (120U/ml, 20min) was used as a positive control. Non-treated and Cys-treated HUVEC from the same donor were used in each experiment as negative controls. 30µg protein from each sample was used in westernblot. Phosphorylated c-Jun was detected using specific antibodies and was identified as a band of approximately 48kD. B: Density of the bands represented in Figure 36A and from 2 further experiments with independent samples were quantified using ImageJ software. These graphs represent the results for non-treated and DL-Hcy alone or with NAC-treated HUVEC which are expressed as mean (+/- SD) from 3 different experiment (n= 3). Non-treated values were defined as 100% and other values were adjusted accordingly. (*) $p < 0.05$ for DL-Hcy versus non-treated cells, (**) $p < 0.05$ for DL-Hcy versus DL-Hcy/NAC-treated cells.

4.2.4. The effects of antioxidants on DL-Hcy-induced HUVEC:neutrophil interactions

Since oxidative stress activates JNK, this may be the sole mechanism by which adhesion molecule expression is up-regulated and therefore, antioxidants may prevent adhesion molecule up-regulation. In order to investigate this possibility, the effects of antioxidants and Hcy on EC:neutrophil interactions were determined as a measure of dynamic adhesion molecule expression. Results in Chapter 2 demonstrated that 1mM DL-Hcy alone induced significant increases in all different sorts of interactions between EC and neutrophils. Therefore, this chapter investigates whether these effects are mediated by ROS. The flow experiments were repeated using two different antioxidants, NAC and vitamin C, in order to confirm that any effects were due to the ROS scavenging properties of each antioxidant.

4.2.4.1. NAC

In the first set of experiments, different concentrations of NAC (1mM, 5mM and 10mM) were used along with DL-Hcy and Figure 37 shows the effects of all of these different treatments. NAC (1mM) did not have measurable reduction on DL-Hcy induced HUVEC:neutrophil interactions while 10mM dramatically blocked all interactions and reduced interactions to below non-treated control level which might mean that NAC at such high concentration might have affected the cellular response to stimuli. 5mM NAC inhibited the effects of DL-Hcy on HUVEC:neutrophil interactions. Although different concentrations of NAC were used by other studies ranging from 1-30mM in cultured EC cell lines (Gu *et al.*, 2002; Xia *et al.*, 2006), 5mM NAC was used in all of the following experiments in this chapter.

Compared to non-treated cells, cells treated with DL-Hcy (1mM) showed a significant increase in their interactions with flowing neutrophils (already described in Chapter 2). When HUVEC were treated with DL-Hcy (1mM) and NAC (5mM), the numbers of tethered, rolled, fixed and transmigrated neutrophils decreased significantly to a level similar to the numbers of interactions in negative control cells (Figure 38). Cells treated with NAC (5mM) alone showed similar numbers of interactions to non-treated control (data not shown).

The effects of different doses of NAC on DL-Hcy-induced interactions (NAC dose response)

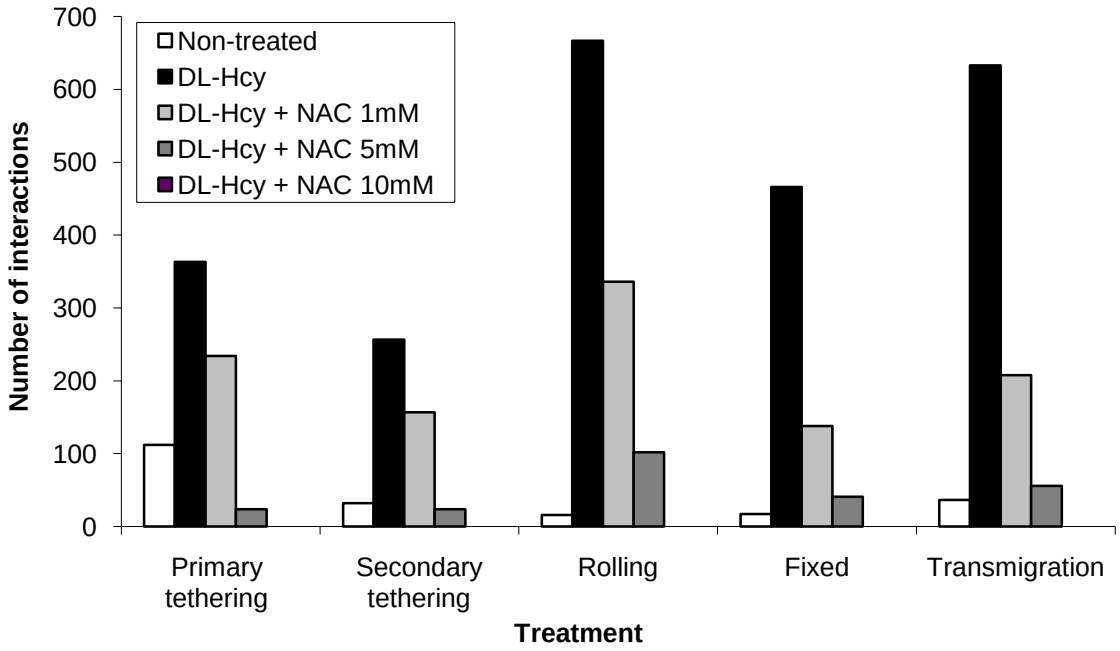


Figure 37: HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) alone or in combination with different concentrations of NAC (1mM, 5mM or 10mM) and the effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in complete M199 media without any stimulation and were considered as negative control in each experiment (n=1).

The effects of antioxidants on DL-Hcy-induced interactions (NAC)

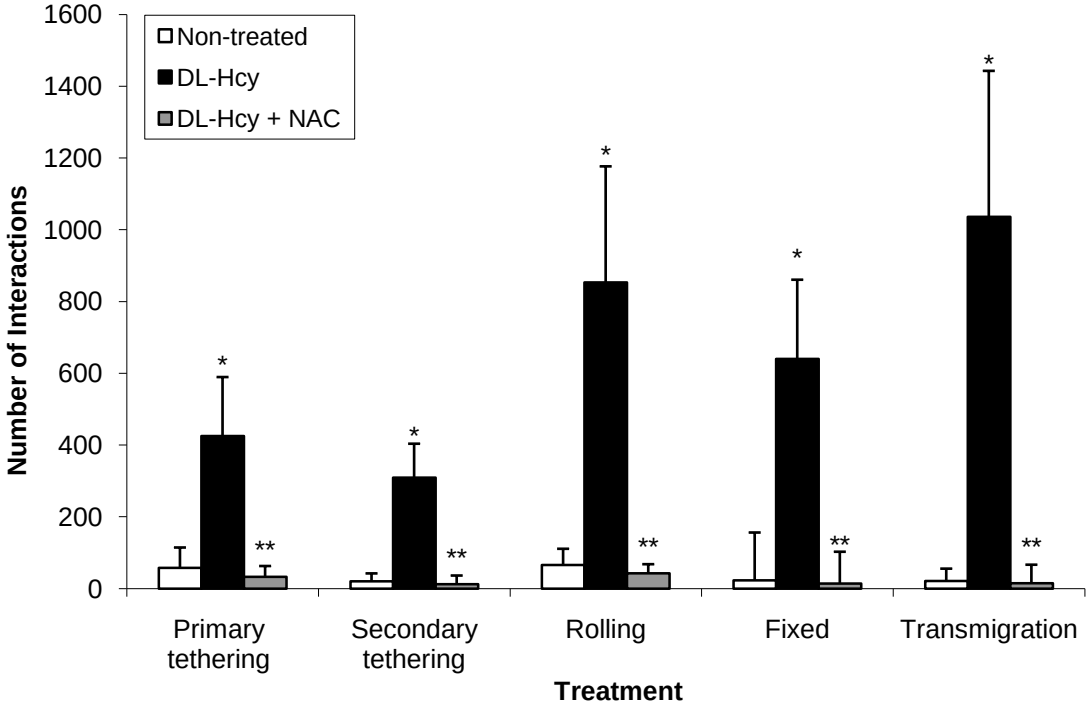


Figure 38: HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) alone or with 5mM NAC and the effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in media without any stimulation and were considered as negative control in each experiment. The results are expressed as mean (+/- SD) from 6 different donors (n=6). (*) $p < 0.001$ for DL-Hcy versus non-treated cells, (**) $p < 0.001$ for DL-Hcy versus DL-Hcy/NAC treated cells.

4.2.4.2. Vitamin C

Vitamin C was used at concentration of 100μM as this concentration has been previously used as an antioxidant by a number of different groups (Ryu *et al.*, 2007; Takaishi *et al.*, 2003). Compared with non-treated cells, cells treated with DL-Hcy showed a significant increase in their interactions with flowing neutrophils (as already described in Chapter 2). When HUVEC were treated with DL-Hcy and vitamin C, the numbers of tethered, rolled, fixed and transmigrated neutrophils decreased significantly to a level similar to the numbers of interactions in negative control cells (Figure 39).

The effects of antioxidants on DL-Hcy-induced interactions (vitamin C)

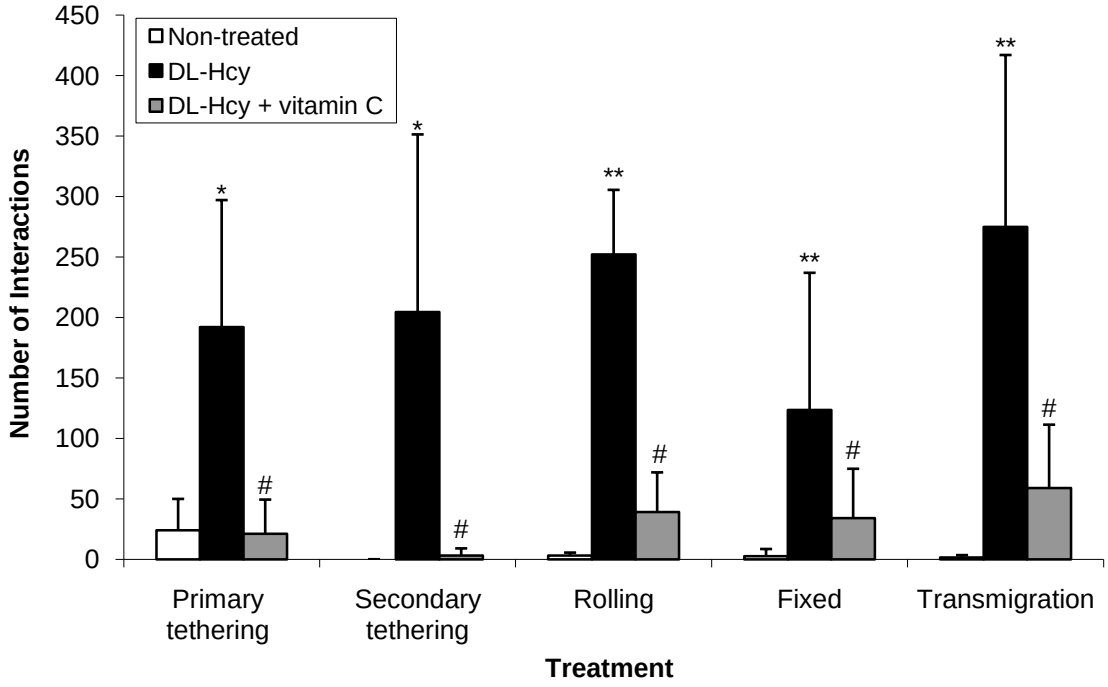


Figure 39: HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) alone or with 100µM vitamin C and the effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in media without any stimulation and were considered as negative control in each experiment. The results are expressed as mean (+/- SD) from 4 different donors. (*) $p < 0.05$ and (**) $p<0.01$ for DL-Hcy versus non-treated cells, (#) $p< 0.05$ for DL-Hcy versus DL-Hcy/vitamin C treated cells (n=4).

4.3. DISCUSSION

Generation of oxidative stress is the most accepted theory to account for Hcy effects or at least a major part of them (Papatheodorou and Weiss, 2007; Weiss *et al.*, 2003). These reviews have listed many studies which investigated different possible pathways through which Hcy induces oxidative stress in the endothelium which leads to atherosclerosis initiation.

Different methods reviewed by Tarpey and Fridovich (2001) have been used and published to detect and measure ROS production in the cells. However, measuring vascular production of ROS is a difficult process because intracellular steady-state $O_2^{\bullet-}$ tends to be very low due to the continuous scavenging by cytoplasmic and mitochondrial SOD. Furthermore, some of this $O_2^{\bullet-}$ might leak to extracellular spaces via anion channels (Rosen and Freeman, 1984). Therefore, the relatively short half-life (seconds) of ROS in addition to the efficient systems involved in scavenging them, require techniques to measure intracellular ROS to be very efficient and sensitive (Tarpey and Fridovich, 2001). Therefore, in this chapter, different direct and indirect methods were used to detect whether chronic treatment with DL-Hcy induces oxidative stress. NBT reduction has been used by several studies to measure the production of superoxide anions (Au-Yeung *et al.*, 2004; Eligini *et al.*, 2005; Patterson *et al.*, 1999) which all indicated that the amount of formed formazan was proportional to the concentration of superoxide anions. Superoxide anions reduce NBT to monoformazan (NBT^+) which can be detected spectrophotometrically. This chapter's results indicate that non-treated and differently treated cells show detectable levels of superoxide anions. These data are in line with other studies which proposed that intact EC express small amount of superoxide anions which play an essential role as a

second messenger mediating different signalling pathways (Kunsch and Medford, 1999; Ullrich and Bachschmid, 2000; Zhang and Gutterman, 2007). Even though all cells showed detectable levels of superoxide anions, DL-Hcy-treated cells showed higher levels (1.5-fold) compared to non-treated cells. This increase was significant ($p<0.01$), indicating that DL-Hcy induced an increase in superoxide anion generation intracellularly. This data match with Au-Yeung *et al* (2004) who used NBT reduction and found that treating HUVEC with 100μM Hcy for 15min induced 1.5-fold increase in intracellular superoxide anions in spite of using only 100μM Hcy. However, in this chapter the generation of superoxide anions was studied after chronic treatment with DL-Hcy (5-9 days) which induced sustained intracellular production of superoxide anions and therefore, the high concentration of DL-Hcy used in this chapter compared to Au-Yeung's study might be required to overcome cellular antioxidant systems and therefore to induce the sustained production of the anions. In contrast, Cys had no effects on producing superoxide anions and their level was similar to non-treated cells indicating that the effect was specific to Hcy and not due to any osmotic effects or non specific due to the free thiol group. The NBT reduction assay could have been done with and without including SOD which would have made the results more relevant and reflect only the superoxide generation. To confirm these findings, a specific nonfluorescent probe DCFH-DA was used to detect the generation of ROS which, if present oxidize DCFH-DA into its fluorescent derivative DCF. DCFH-DA was shown to be oxidized by different ROS like H_2O_2 in BAEC (Royall and Ischiropoulos, 1993; Tampo *et al.*, 2003) and by oxidants produced during the respiratory burst in inflammatory cells (Tarpey and Fridovich, 2001). Photos in Figure 35 show that non-treated cells and Cys-treated cells showed a very low level of fluorescence compared to DL-Hcy-treated cells.

These results clearly indicate that DL-Hcy treatment induced ROS accumulation and thus oxidative stress in HUVEC. These findings match those of Tyagi *et al* (2005) who used the same probe DCFH-DA and found significant increases in ROS generation in Hcy-treated rat cardiac microvascular EC (40μM Hcy for 24h).

The DCFH-DA probe is very sensitive to aerobic conditions so stock solutions were purged with nitrogen immediately after preparation and stored in -80°C. However, within a few days, the dye was bleached and stopped working. Even though different studies managed to get reproducible results using this probe (Tampo *et al.*, 2003; Tyagi *et al.*, 2005), and despite using two different batches of the dye from two different suppliers (Sigma Aldrich and Calbiochem) in this chapter, it was very hard to get reproducible results. Therefore, the photos in Figure 35 represent only two experiments. Collectively, the results of the NBT reduction assay and DCFH-DA probe together give strong evidence that chronic treatment with DL-Hcy induced oxidative stress in HUVEC. These findings match with previously published studies that confirmed Hcy-induced oxidative stress in vascular EC using different techniques: both Handy *et al* (2005) and Upchurch *et al* (1997) showed that Hcy induces dose dependent inhibition of glutathione peroxidase in cultured BAEC and thus accumulation of H_2O_2. Zhang *et al* (2000) also demonstrated that 50μM Hcy for 24h induced significant generation of ROS in HUVEC. Furthermore, patients with acute coronary syndromes showed elevated levels of Hcy and malondialdehyde, a marker for oxidative stress, in addition to decreased expression of SOD (Wang *et al.*, 2004). However, there is still controversy as an early study reported that Hcy does not induce oxidative stress but induces reductive stress which leads to ER stress and induces EC damage (Outinen *et al.*, 1998). These authors built their conclusion on their findings that Hcy (1-5mM for 18h)

was not able to induce expression of heat shock proteins but induced expression of stress response genes which are known to be induced by reducing agents in HUVEC. However, they showed also that Hcy inhibited the expression of antioxidant enzymes like glutathione peroxidase which enhances the cytotoxic effects of ROS. Other techniques could have been used to establish which species of ROS are mediating Hcy-induced oxidative stress such as L-band electron spin resonance with nitroxyl probes and magnetic resonance imaging spin trapping (Halliwell and Whiteman, 2004).

The next set of experiments were designed to detect whether scavenging DL-Hcy-induced ROS by antioxidants would disrupt DL-Hcy effects on JNK pathway and the activation of the JNK substrate c-Jun, in addition to characterizing the effects of antioxidant treatment on DL-Hcy-induced HUVEC:neutrophil interactions. Firstly and to check the efficiency of NAC as a ROS scavenger, DCFH-DA dye was used. Figure 35 indicates that cells treated with DL-Hcy/NAC-treated cells show a very low level of fluorescence compared to DL-Hcy-treated cells which indicates that NAC was an efficient ROS scavenger and was able to overcome DL-Hcy-induced ROS production. DL-Hcy has already been shown in Chapter 3 to activate the JNK pathway and subsequently to induce phosphorylation of its substrate c-Jun leading to up-regulation of CAM. However, once these cells were treated with DL-Hcy and NAC, JNK pathway activation was blocked and c-Jun was not phosphorylated (Figure 36) which indicates that NAC inhibited DL-Hcy effects on JNK pathway. It is likely that NAC scavenging of ROS inhibited DL-Hcy-induced JNK activation. The involvement of ROS in mediating MAP kinase pathway activation has been suggested in VSMC in response to angiotensin II (Ushio-Fukai *et al.*, 1998) and in neutrophils in response to Hcy (Alvarez-Maqueda *et al.*, 2004). Our lab is the

first to demonstrate that ROS play a role in mediating chronic Hcy-induced MAPK JNK in EC (Alkhoury *et al.*, 2009b). NAC (20mM) also blocked TNF-α-induced activation of JNK1 in human monocyte cell line (U937) (Gu *et al.*, 2002). Furthermore, ROS in fibroblasts (Kamata *et al.*, 2005) and in human embryonic kidney 293 cells (Chen *et al.*, 2001a) were reported to inhibit JNK phosphatase, which is responsible for dephosphorylating activated JNK, and therefore ROS induced sustained activation of JNK. Results in Chapter 3 clearly showed that chronic DL-Hcy induced sustained activation of JNK (Figures 27 and 28) and the current results (Figure 36) show that scavenging ROS by NAC inhibited this JNK activation indicating that ROS mediate Hcy-induced JNK activation under static conditions in HUVEC in culture. One study found that 20mM NAC (1h pre-treatment followed by 4h of 3mM Hcy) was not able to reverse Hcy-induced JNK activation in HUVEC (Cai *et al.*, 2000). The high concentration of Hcy used in this study might have induced huge ROS production that was not able to be scavenged totally with NAC and that might explain why NAC was not able to reverse Hcy-induced JNK activation.

In this chapter, treatment with antioxidants NAC (Figure 38) or vitamin C (Figure 39) managed to reverse the effects of DL-Hcy on HUVEC:neutrophil interactions under flow and the number of all different sorts of interactions were similar to those of non-treated cells. These effects of antioxidants are definitely due to their ROS scavenging properties as the results were reproducible with two different antioxidants, NAC and vitamin C. That means ROS mediate the DL-Hcy effects on HUVEC:neutrophil interactions under flow and that they are involved in the signalling pathways that lead to up-regulation of different CAMs and/or chemokines which mediate different types of

interactions between EC and leukocytes. This chapter provides clear evidence that chronic treatment with Hcy induced EC dysfunction by a ROS-mediated mechanism that is characterized by activation of the JNK pathway and increased EC:neutrophil interactions under flow conditions which all were reversible by antioxidant treatment. Vitamin C has been previously used successfully in a few studies and managed to reverse Hcy-induced EC dysfunction (Chambers *et al.*, 1999; Nappo *et al.*, 1999) and to reverse Hcy coronary effects in patients with experimentally induced hHcy (Yamashita *et al.*, 2005). NAC and vitamin C also reversed Hcy inhibitory effects on L-arginine intracellular uptake in BAEC (Jin *et al.*, 2007). This chapter is the first study to confirm that ROS accounts for most Hcy effects on EC *in vitro* in addition to the efficiency of antioxidants in reversing these effects under static and flow conditions.

Tyagi (1998) showed, using fluorescence labelled homocystine, that homocystine binds to receptors on human vascular SMC membrane and some of this homocystine managed to become intracellular. However, the group could not explain whether results were due to diffusion or transport of homocystine into the cell through receptor internalization or redox channel mechanisms. On the other hand, Tyagi showed that NAC reversed homocystine binding but they could not explain whether these reversing effects were due to antioxidant activity of NAC or due to interaction between the two compounds which might prevents homocystine from binding to its proposed receptors (Tyagi, 1998). Furthermore, Ewadh *et al* (1990) showed that including Cys in the media inhibited Hcy cellular uptake by HUVEC and they suggested this inhibition to be due to the formation of mixed disulphides between Hcy and Cys. NAC has a free thiol group similar to Hcy and Cys, which is readily available to form disulphide bonds with other thiol containing groups.

Data provided by both Tyagi and Ewadh *et al* suggest that NAC might interact with Hcy forming mixed disulphides thus preventing Hcy intracellular uptake. Thus, presenting another possible mechanism for NAC by which it inhibits Hcy effects in addition to its ROS scavenging activity. Moreover, further studies could be used to detect the presence of such mixed disulphides by using techniques, such as mass spectroscopy (MS). Preliminary data using MS (appendix 3) showed that Hcy and NAC might interact to form a mixed disulphide. However, this intermediate compounds is likely to be instable in physiological pH. Further studies are required to optimize a protocol using the appropriate buffer to evaluate the efficiency of NAC in preventing Hcy cellular uptake (appendix 3).

NAC was shown to decrease cell apoptosis and restore balance redox status in a TNF-α-treated EC cell line (ECV304) (Xia *et al.*, 2006) and also was shown to inhibit, in a dose dependent fashion, LDL oxidation *in vitro* (in a test tube) (Rattan and Arad, 1998). Furthermore, NAC inhibited endothelin-1-induced JNK activation in VSMC (Fei *et al.*, 2000) and was shown to inhibit cytokine-induced expression of VCAM-1 and MCP-1 in EC (Kunsch and Medford, 1999). All of these studies indicate that NAC is an effective antioxidant and this data, collectively with the results of this chapter, provide strong evidence that DL-Hcy-induced EC dysfunction and the subsequent chronic inflammatory response are mediated by oxidative stress.

Despite that, a study by Frank *et al* (2007) suggested that Hcy did not induce ROS generation and did not induce cytotoxicity to human microvascular endothelial cell lines. Furthermore, they found that adhesion of monocytes to Hcy-treated EC was not significant and they suggested that Hcy was not able to induce oxidative stress, inflammation or EC dysfunction. Frank's group results contradict those of this chapter and different reasons

might account for this contradiction. Firstly, they used endothelial cell lines which might respond in a different way from primary EC to Hcy treatment and they might be more resistant to Hcy treatment. Secondly, they exposed EC to Hcy for only 4h which may not enough to induce significant expression of some CAM such as ICAM-1 whose expression requires 6-12h to peak and which is essential for monocyte adhesion. Finally, they carried out their adhesion assay under static conditions where involvement of NO generated under shear stress cannot be taken into account and is therefore less relevant to the *in vivo* situation.

Although different *in vitro* studies have provided evidence of the efficiency of antioxidants in reducing the inflammatory conditions and to have anti-atherosclerotic effects (Gu *et al.*, 2002; Kamata *et al.*, 2005; Kyaw *et al.*, 2004; Rattan and Arad, 1998; Siow *et al.*, 1998; Xia *et al.*, 2006), several clinical studies have failed to provide clear evidence of the benefits of antioxidants in reducing risk of different cardiovascular diseases (Paolini *et al.*, 2003; Stanner *et al.*, 2004) as already highlighted in section 1.3.4. It is still unclear why antioxidants clear beneficial effects *in vitro*, while less satisfying results were observed in clinical studies (Kyaw *et al.*, 2004).

The mechanism by which Hcy induces ROS overproduction is not clear and this chapter does not include any experiments to attempt to resolve this. However, other studies have suggested and provided evidence of different mechanisms which could mediate Hcy-induced ROS production. NADPH oxidase is the major source of $O_2^{\bullet -}$ in EC (Ullrich and Bachschmid, 2000) and different studies have provided evidence of Hcy-induced activity of NADPH oxidase (Dong *et al.*, 2005; Ungvari *et al.*, 2003). Furthermore, Dong's group showed that inhibition of NADPH oxidase with apocynin prevented Hcy-induced ROS

generation. Other researchers believe that eNOS under certain conditions might produce $O_2^{\bullet -}$ in the expense of NO (this hypothesis will be discussed in more details in Chapter 5). NADPH oxidase could be upstream of other ($O_2^{\bullet -}$)-producing enzymes because superoxide anions produced by NADPH oxidase appear to be implicated in the activation of other $O_2^{\bullet -}$ producing enzymes like eNOS uncoupling (Landmesser *et al.*, 2003).

4.4. CONCLUSION

This chapter demonstrates that Hcy induces oxidative stress in EC and this is required for their activation. This has been indicated by JNK activation with the subsequent functional effects of increasing EC:neutrophil interactions under flow which indirectly indicates downstream CAM expression. Furthermore, this chapter presents clear evidence of the efficiency of two different antioxidants in protecting EC from Hcy damage, implicating oxidative stress as the mechanism by which HUVEC are activated in response to chronic Hcy.

CHAPTER 5

5. THE ROLE OF NITRIC OXIDE AND NITRIC OXIDE SYNTHASE IN HCY-MEDIATED ATHEROGENIC EFFECTS

The health of the vascular system depends on the balance between anti-inflammatory and anti-thrombotic nitric oxide together with levels of pro-inflammatory superoxide and other radicals like peroxynitrite, and the hydroxyl radical (reviewed by Forstermann (2008), Naseem (2005) and Tomasian *et al* (2000). Disturbances to this balance, leading to reduced nitric oxide (NO) bioavailability result in induction of a pro-inflammatory phenotype within endothelial cells (EC) lining the blood vessels, with up-regulation of CAM and chemokine production. Recruitment of leukocytes which contribute to atherosclerotic plaque development then occurs (Lusis, 2000).

It is known that a lack of either the substrate L-arginine or the cofactor BH_4 leads to uncoupling of the eNOS leading to superoxide anion production at the expense of NO (Xia *et al.*, 1998). Hcy has been suggested to induce eNOS uncoupling either via reducing levels of BH_4 (Zhang *et al.*, 2000) or decreasing intracellular uptake of L-arginine (Jin *et al.*, 2007). Therefore, uncoupled eNOS could represent a major source of ROS in Hcy-treated EC.

It is clear from the previous chapters that chronic Hcy induced sustained oxidative stress inducing prolonged activation of the JNK pathway and therefore EC activation. Furthermore, scavenging ROS by different antioxidants reverseed all of these effects. However, the questions which remain unanswered are how DL-Hcy induces ROS

generation and would restoring NO bioavailability restore the EC quiescence. Therefore, this chapter investigated the possibility of uncoupled NO as a major source of ROS in chronically treated Hcy. Furthermore, we investigated the efficiency of restoring NO bioavailability on reversing Hcy-induced HUVEC:leukocyte interaction.

5.1. MATERIALS and METHODS

5.1.1. Materials

Two NO donors were used GSNO (0603) was purchased from Tocris biosciences (Bristol, UK) and DPTANONOate (ALX430066) was purchased from Alexis Biochemicals. Two eNOS inhibitors were used, L-NAME (483125) and L-NIO (400600) were purchased from calbiochem (La Jolla, USA). ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one) (495320) was also purchased from Calbiochem, (La Jolla, USA). DL-dithiothreitol (D-0632) was purchased from Sigma-Aldrich (Poole Dorset, UK).

5.1.2. HUVEC culture and treatment

5.1.2.1. NO donors

HUVEC were cultured in the presence of DL-Hcy chronically as in section 2.1.2.2 and then subjected to flow as described in section 2.1.2.7. However, 10min prior to flow, DL-Hcy-treated cells were exposed to either GSNO (20µM) or DPTA (20µM). Furthermore, some of the DL-Hcy treated cells were pre-treated with 40µM ODQ for 30min followed by treatment with 20µM GSNO for 10min. Subsequently, these cells were subjected to flowing neutrophils as explained in section 2.1.2.7. Some HUVEC (DL-Hcy-treated and non-treated cells) were also treated with different concentrations of the reducing reagent DTT (0.1mM and 1mM) for 20min prior to flow or prior to NO donor treatment.

4.7.2.2. eNOS inhibitors

HUVEC were cultured for a period of 5-9 days in complete M199 medium containing 1mM DL-Hcy alone or with 1mM L-NAME. HUVEC were then subjected to flow as described in section 2.1.2.7. The same experiments were repeated with another

eNOS inhibitor L-NIO (1mM). Furthermore, NBT reduction was used to measure superoxide anions generation in the treated-cells as described in section 4.1.3.1.

5.2. RESULTS

5.2.1. NO reverses DL-Hcy-induced HUVEC:neutrophil interactions

To study the effects of DL-Hcy on NO bioavailability and whether restoring NO bioavailability might reverse the effects of DL-Hcy on EC:neutrophil interactions, two NO donors were used separately to flood the EC with NO. As before (section 2.2.3.6) DL-Hcy significantly induced EC:neutrophil interactions. Despite the short incubation period (10min), DPTANONOate was able to reverse all types of HUVEC:neutrophil interactions: tethering, rolling, adhesion and transmigration, to levels similar to those of non treated cells (Figure 40A). The difference between numbers of interactions of DL-Hcy alone treated cells and those pre-treated with DPTA were significant ($p<0.05$). Similar results were obtained with the second NO donor GSNO (Figure 40B). Some DPTA was exposed to light at room temperature to stop its ability to generate NO. Therefore, this "broken" DPTA was used as a negative control to make sure that the observed effects were due to NO and not to any chemical property of the NO donor. "Broken" DPTA was not able to reverse the effects of DL-Hcy on HUVEC:neutrophil interactions (data not shown). Furthermore and to study whether these effects were occurring via a cGMP mediated pathway, the cGMP inhibitor ODQ was used to inhibit this pathway prior to treatment with NO donors. Inhibiting cGMP caused no alteration to the effects of the NO donor in reversing DL-Hcy-mediated interactions. Cells treated with DL-Hcy/GSNO/ODQ showed significant decreases in their interactions with neutrophils to a level similar to those treated with DL-Hcy/GSNO and to the non-treated cells (Figure 41).

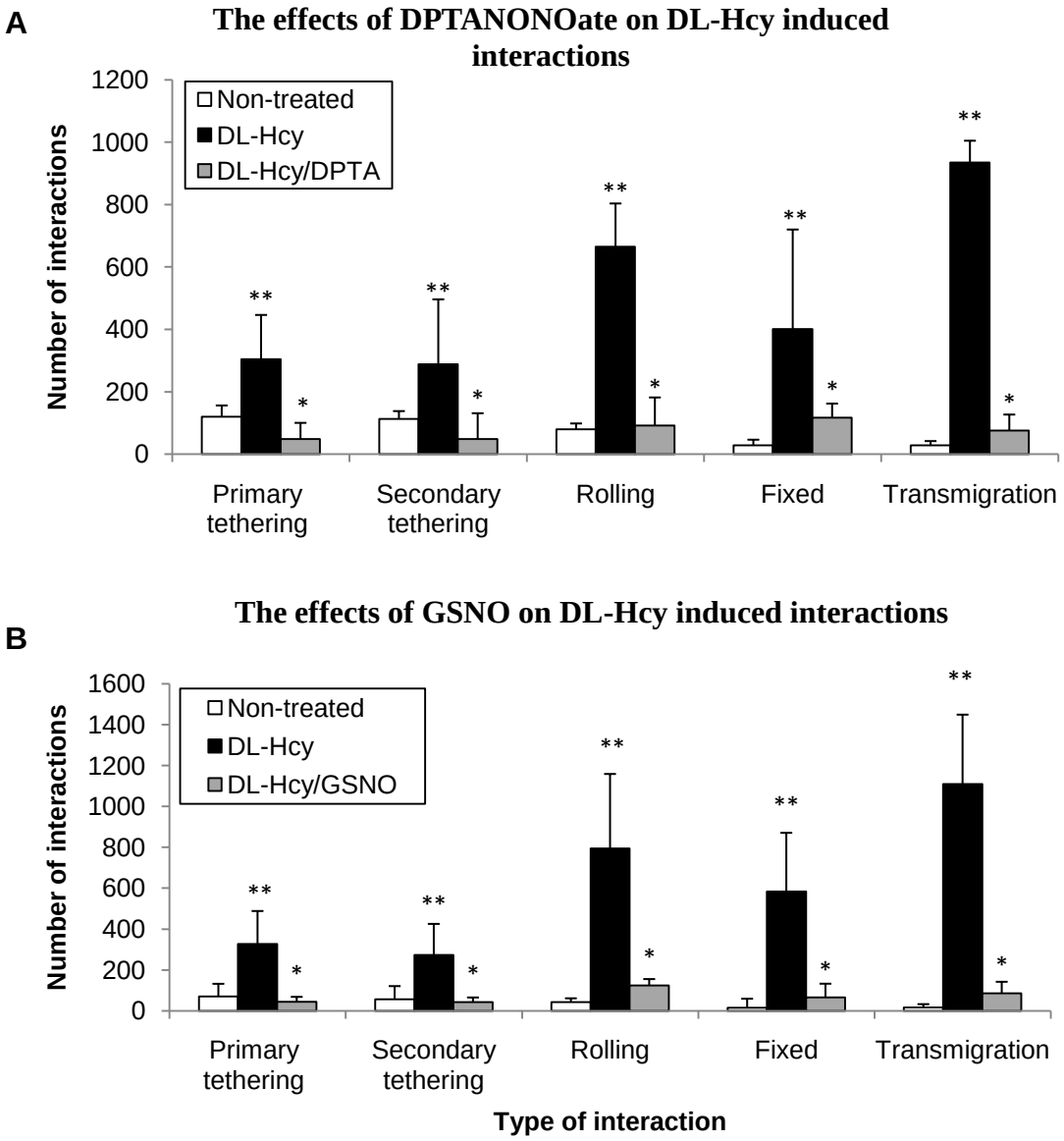


Figure 40: The effects of the NO donors DPTA (A) or GSNO (B), on HUVEC:neutrophil interactions. HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) and some of these DL-Hcy-treated cells were exposed to DPTA (20µM) or GSNO (20µM) for10min and the effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in media without any stimulation and were considered as negative controls in each experiment. The results are expressed as mean (+/- SD) from 3 different donors for DPTA and 6 different donors for GSNO. (*) $p < 0.05$ for DL-Hcy versus DL-Hcy/NO donor treated cells, (**) $p < 0.05$ for DL-Hcy versus non-treated cells.

The role of cGMP in NO donor effects on DL-Hcy-induced interactions

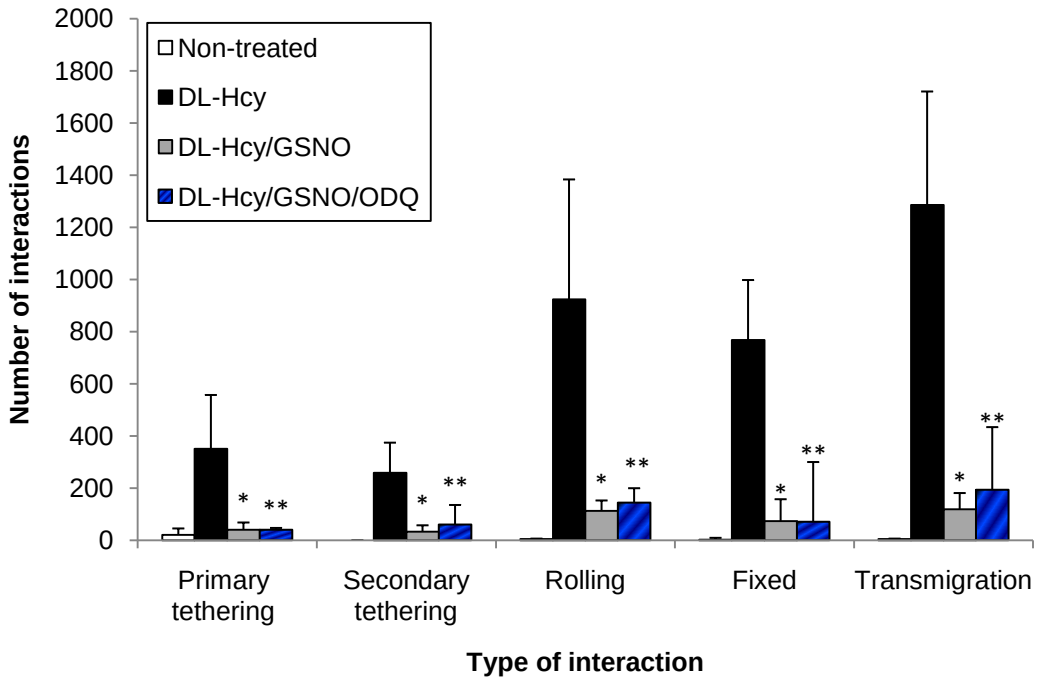


Figure 41: HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) and some of these DL-Hcy-treated cells were pre-treated with 40µM ODQ for 30min followed by treatment with 20µM GSNO for 10min. The effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in media without any stimulation and were considered as negative controls in each experiment. The results are expressed as mean (+/- SD) from 3 different donors. (*) $p < 0.05$ for DL-Hcy versus DL-Hcy/GSNO treated cells, (**) $p < 0.05$ for DL-Hcy treated cells versus DL-Hcy/ODQ/GSNO treated cells.

5.2.2. The effects of reducing agents on DL-Hcy-stimulated HUVEC:neutrophil interactions

NO was shown in the previous section to inhibit DL-Hcy-induced HUVEC:neutrophil interactions under flow via a cGMP-independent mechanism. Nitrosylation of some proteins might explain these effects of NO treatment. Reducing reagents like DTT have previously been shown to reverse nitrosylation of some proteins (Park *et al.*, 2006; Park *et al.*, 2000a). Therefore, DTT was used in this chapter to check whether it could reverse NO effects on DL-Hcy-treated cells. Firstly, DTT effects on non-treated and DL-Hcy-treated HUVEC were investigated. In the first experiment, HUVEC were treated with 1mM DTT alone for 20min and then subjected to flowing neutrophils. These cells did not interact with the flowing neutrophils (Figure 42), and furthermore, cell phenotype was affected and they lost their normal phenotype. Following that, DL-Hcy-treated cells were treated with either 1mM or 0.1mM DTT for 20min before being subjected to flowing neutrophils. DTT appeared to have a dose-dependent response on the cells and number of interactions decreased compared to DL-Hcy-treated cells. 1mM DTT reduced HUVEC:neutrophil interactions more than 0.1mM DTT (Figure 42). However, HUVEC in both cases looked abnormal as well and lost their normal cobblestone arrangement.

The effects of reducing reagents (DTT) on DL-Hcy-induced interactions

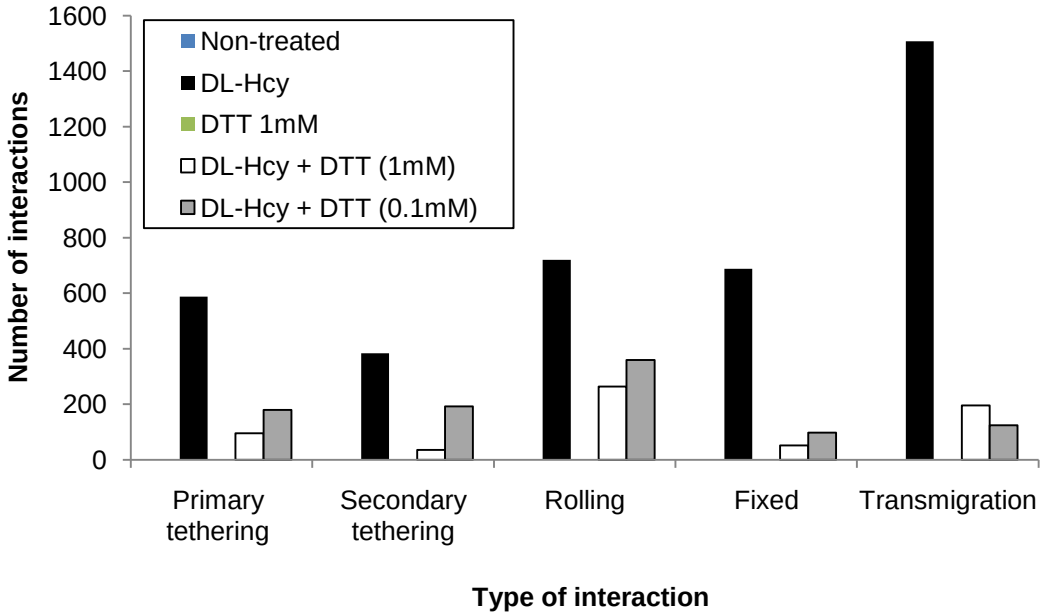


Figure 42: HUVEC (1mM DL-Hcy-treated and non-treated cells) were also treated with different concentrations of the reducing reagent DTT (0.1mM or 1mM) for 20min prior to flow. These results represent only one experiment for each concentration of DTT.

5.2.3. The role of eNOS in DL-Hcy-induced effects

DL-Hcy was shown previously (Chapter 4) to induce ROS generation in EC which mediated DL-Hcy-induced EC:neutrophil interactions however, the source of these ROS is still unclear. Uncoupled eNOS represent a major source of ROS and therefore this chapter investigates the role of eNOS in mediating the DL-Hcy-induced oxidative stress in HUVEC and whether it is responsible for generating superoxide anions at the expense of NO. eNOS in HUVEC was inhibited and then the consequences of such inhibition were studied under static and flow conditions.

5.2.3.1. Effects of inhibition of eNOS on superoxide generation

NBT reduction was used to measure the generation of intracellular superoxide anions in differently treated cells (as explained in section 4.1.3.1). As already demonstrated in section 4.2.1, 1mM DL-Hcy induced significant generation of superoxide anions. However, once these cells were treated with 1mM DL-Hcy and 1mM L-NAME together, superoxide anion production decreased significantly (Figure 43) ($p<0.05$). L-NAME alone had no effects on superoxide anion production with levels generated being similar to that in non-treated cells.

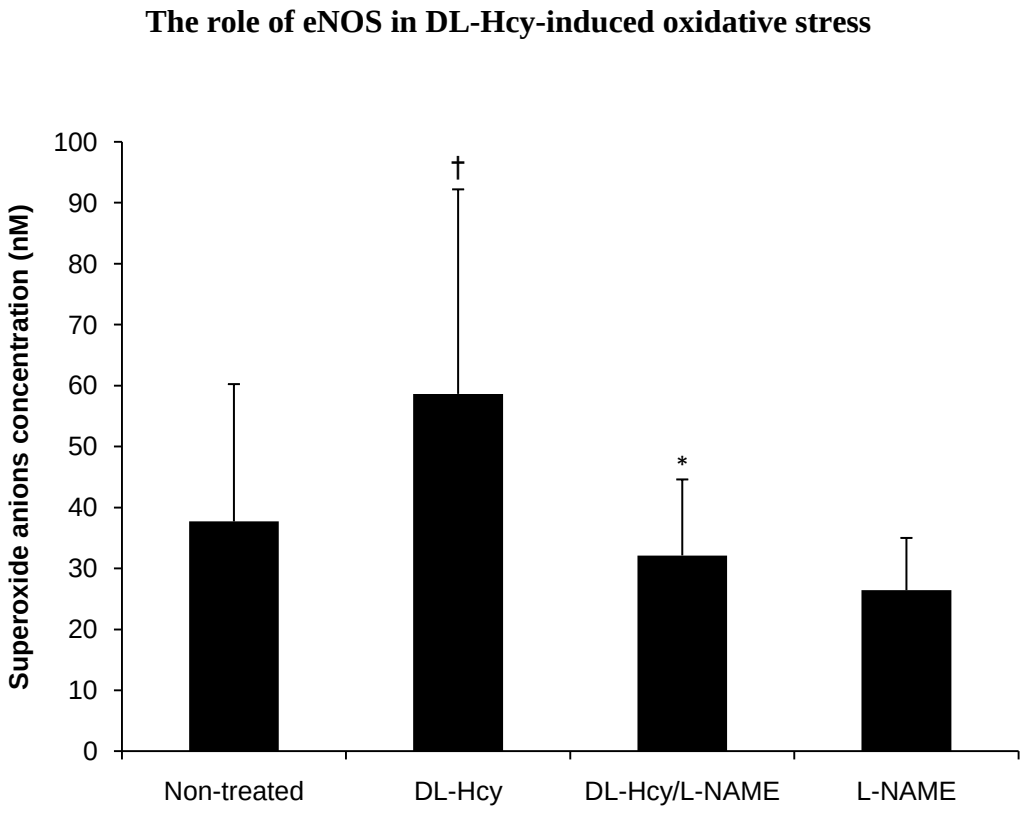


Figure 43: HUVEC were grown in complete medium and either not treated or treated with DL-Hcy (1mM) alone or with L-NAME (1mM) for 5-9 days. Non-treated cells were considered as negative controls. Media was replaced with phenol red-free media containing NBT (1mg/ml) and incubated at 37°C and 5% CO_2 for 90min. Cells were lysed in pyridine, heated at 90°C for 20min and absorbance was measured immediately at 570nm. Extinction coefficient: 7200 $M^{-1}xcm^{-1}$. The results are expressed as mean (+/- SD) from 7 different donors (n=7). (†) $p < 0.05$ for DL-Hcy versus non-treated cells. (*) $p < 0.05$ for DL-Hcy versus DL-Hcy/L-NAME-treated cells.

5.2.3.2. The role of eNOS in DL-Hcy-induced HUVEC:neutrophil interactions

As eNOS was shown in the previous section to be responsible for producing ROS in response to chronic DL-Hcy treatment, the effects of inhibiting eNOS on DL-Hcy-induced HUVEC:neutrophil interactions was investigated. HUVEC were treated with DL-Hcy with and without the specific eNOS inhibitor L-NAME (1mM) for 5-9 days and then subjected to flowing neutrophils. As before (section 2.2.3.6) DL-Hcy induced significant HUVEC:neutrophil interactions. L-NAME managed to reverse all the effects of DL-Hcy ($p<0.05$) and numbers of tethering, rolling, adhesion and transmigration interactions of DL-Hcy/L-NAME-treated cells were similar to those of non-treated cells (Figure 44). These results were reproducible with another eNOS inhibitor L-NIO (1mM) (data not shown). L-NAME alone showed not to have any effects on HUVEC:neutrophil interactions.

The effects of eNOS inhibition on DL-Hcy-induced interactions

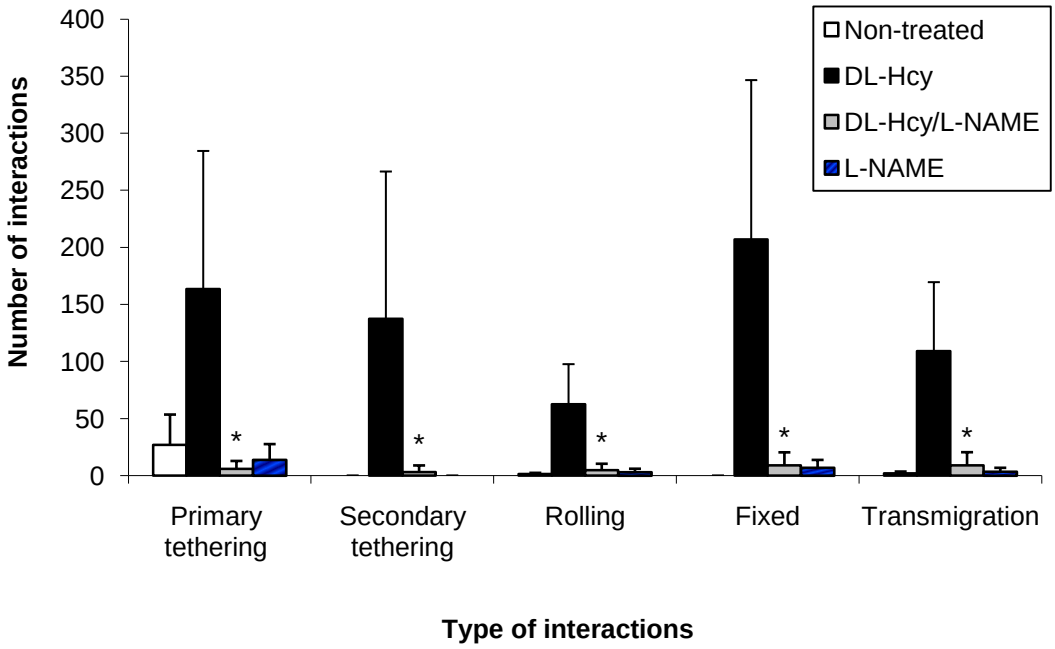


Figure 44: HUVEC were stimulated with DL-Hcy (1mM; 5-9 days) alone or with 1mM L-NAME and the effects on EC:neutrophil interactions under flow conditions were observed and quantified. HUVEC from the same donor were grown in media without any stimulation and were considered as negative controls in each experiment. The results are expressed as mean (+/- SD) from 4 different donors (n=4). (*) $p < 0.05$ for DL-Hcy versus DL-Hcy/L-NAME treated cells.

5.3. DISCUSSION

The importance of nitric oxide in CVD has been investigated intensively and NO has been strongly implicated in protecting against the initiation and progression of different CVD (reviewed by Naseem (2005)). NO is also involved in regulating different CAM expression on EC and leukocytes (Takahashi *et al.*, 1996). Furthermore, NO can inhibit LDL oxidation by reacting with lipid peroxyl radicals (Rubbo *et al.*, 1994). Collectively it suppresses the inflammatory response via these effects. Hcy as a risk factor in CVD has been demonstrated in published work and in the data obtained in Chapter 4 of this thesis since it induces oxidative stress which mediates inflammatory interactions between activated EC and neutrophils. eNOS uncoupling might be mediating these effects in hHcy patients through generating superoxide. Therefore, experiments in this chapter were designed to study whether restoring NO bioavailability might reverse Hcy inflammatory effects and also to investigate whether Hcy induces eNOS uncoupling which could account for the generated oxidative stress.

There is accumulating evidence from the past few decades about the involvement of NO in regulating CAM expression but most of these studies have used exogenous donors for the same period of CAM-inducing stimuli (Kosonen *et al.*, 2000; Spiecker *et al.*, 1998; Takahashi *et al.*, 1996; Zampolli *et al.*, 2000). Cytokine treatments in all the previous studies were acute (up to 12h) and therefore they used the NO donors for the same length of time as the CAM inducing stimuli. In this chapter, even though NO donor treatment was very short (10min after 5-9 days of DL-Hcy exposure) there was still full reversal of each type of interaction which had been induced by chronic DL-Hcy alone treatment under flow conditions (Figure 40). Two different NO donors were used to determine conclusively that

the effects were due to NO release. The fact that broken DPTA, which is not able to generate NO, was not able to induce similar effects to DPTA further strengthens the evidence. There have been conflicting reports as to whether NO-stimulated effects on CAM are sGC and cGMP dependent (Marshall and Stamler, 2001; Takahashi *et al.*, 1996; Zampolli *et al.*, 2000). To check whether the observed effects of NO happened through a cGMP mediated pathway, cells were treated with the cGMP specific inhibitor ODQ prior to GSNO treatment. ODQ has been suggested to selectively inhibit the haem site of sGC inducing irreversible inactivation of the enzyme and therefore inhibiting cGMP production (Schrammel *et al.*, 1996). However, inhibiting cGMP did not reverse GSNO effects which means all of these effects were occurring through a cGMP-independent pathway (Figure 41). As *de novo* synthesis requires 3h for P-selectin in mice (Weller *et al.*, 1992), 4-8h for E-selectin (Bevilacqua and Nelson, 1993) and up to 12h for ICAM-1 (Dustin *et al.*, 1986), this 10min treatment with NO donors was too short to induce changes at the transcriptional level and to disturb synthesis of any of these adhesion molecules. Therefore, it could be said that the NO-mediated dramatic decrease of DL-Hcy-induced EC:neutrophil interactions was unlikely to be due to inhibition of CAM synthesis.

Within the literature NO donor treatment was shown to have contradicting effects on CAM *de novo* synthesis using different mechanisms. Cytokine-induced ICAM-1 expression in HSVEC (Spiecker *et al.*, 1998) and HUVEC (Takahashi *et al.*, 1996) was inhibited by NO donors in a cGMP-independent manner when cells were treated with NO donor prior to cytokine treatment. Furthermore, NO donor exposure inhibited E-selectin expression after stimulation with cytokines or lipopolysaccharide in both HSVEC and HUVEC (Kosonen *et al.*, 2000; Zampolli *et al.*, 2000). However, Kosonen's and

Zampolli's groups found that pre-treatment with NO donors in HSVEC did not attenuate cytokine-induced ICAM-1 expression and they explained that by the high constitutive expression of ICAM-1 under basal conditions (Kosonen *et al.*, 2000; Zampolli *et al.*, 2000).

Published findings about NO donor effects on P-selectin are more consistent. P-selectin mRNA was increased significantly in human iliac vein EC treated with eNOS inhibitor L-NAME (Armstead *et al.*, 1997) and reached a maximum level after 4h of treatment. The same group used NO donors to demonstrate a significant decrease in mRNA level of P-selectin with a maximum effect after 4h. Furthermore, NO donors were shown to decrease ischemia-reperfusion-induced P-selectin expression in murine endothelial sections (Gauthier *et al.*, 1994). On the other hand another study in HUVEC found that a sGC activator (BAY 41-2272) was able to reverse both IL-1β-induced and histamine-induced P-selectin expression suggesting a NO-independent and cGMP-dependent mechanism instead (Ahluwalia *et al.*, 2004). Collectively, NO seems to play different roles in regulating the expression of different CAM and this regulation seems to happen via different pathways, both cGMP- dependent and independent.

Unlike CAM induction for E-selectin and ICAM-1, P-selectin expression on the cell surface requires the exocytosis of WPB to mobilize P-selectin from its storage vesicles to the surface and this happens within 2min (Hattori *et al.*, 1989). Different protein superfamilies are involved in this process like NSF and SNARE (Lowenstein *et al.*, 2005). NSF is composed of N-terminal domain and two homologous ATP binding domains (D1 and D2). The D1 domain hydrolyzes ATP providing the mechanical force required to disassemble SNARE complex (Matsushita *et al.*, 2003). Matsushita'a group found that some Cys residues in NSF protein are responsible for its interaction with SNARE while

other Cys residues regulate NSF disassembly of SNARE complexes. They also showed that some of these Cys residues could be nitrosylated which will inhibit their activity and therefore inhibit the disassembling of SNARE leading to blocking the exocytosis. NO has been shown to nitrosylate NSF in cultured human EC (Matsushita *et al.*, 2003) and in platelets (Morrell *et al.*, 2005). Furthermore, addition of recombinant NSF to EC pretreated with NO was shown to restore the exocytosis (Matsushita *et al.*, 2003). While, the same group showed that once recombinant NSF was pretreated with NO donor, it was not able to restore WPB exocytosis. Therefore, NO inhibits NSF enzymatic activity by nitrosylation and thereafter inhibits the whole exocytosis process. Subsequently, no P-selectin will be mobilized to the cell surface. Chapter 2 of this thesis showed DL-Hcy induced P-selectin expression and it is suggested this expression continues to cycle once DL-Hcy is present continuously as illustrated in Figure 45.

These steps in the continuous presence of DL-Hcy are happening in a cycle. However, NO inhibits the WPB exocytosis and thus breaks this cycle and the P-selectin which is already present on cell surface will be shed in the first few seconds of exposure of the EC to laminar flow and then cannot be replaced and therefore, the tethering step will be blocked due to lack of P-selectin the main mediator of this step. There is little likelihood of flowing neutrophils starting to roll directly unless they are tethered first on EC to slow them down and allow enough time to start the bond formation between E-selectin and its ligand and also for ICAM-1 and integrins to bind and mediate adherence of the neutrophils. Furthermore, P-selectin has been shown to induce leukocyte activation which is essential for the integrin conformational changes from the non-adhesive form to the adhesive one (Lawrence and Springer, 1991; Lorant *et al.*, 1993). So, it could be concluded that blocking

P-selectin expression and therefore neutrophil tethering led to blockade of all the following steps although other CAM expression might not have been affected by NO treatment. Furthermore, the chemokine IL-8 which is known to play a major role in neutrophil adherence and migration is also stored in WPB. Therefore, blocking WPB exocytosis will induce decreases in IL-8 availability and that will amplify the negative effects of NO on cell:cell interactions.

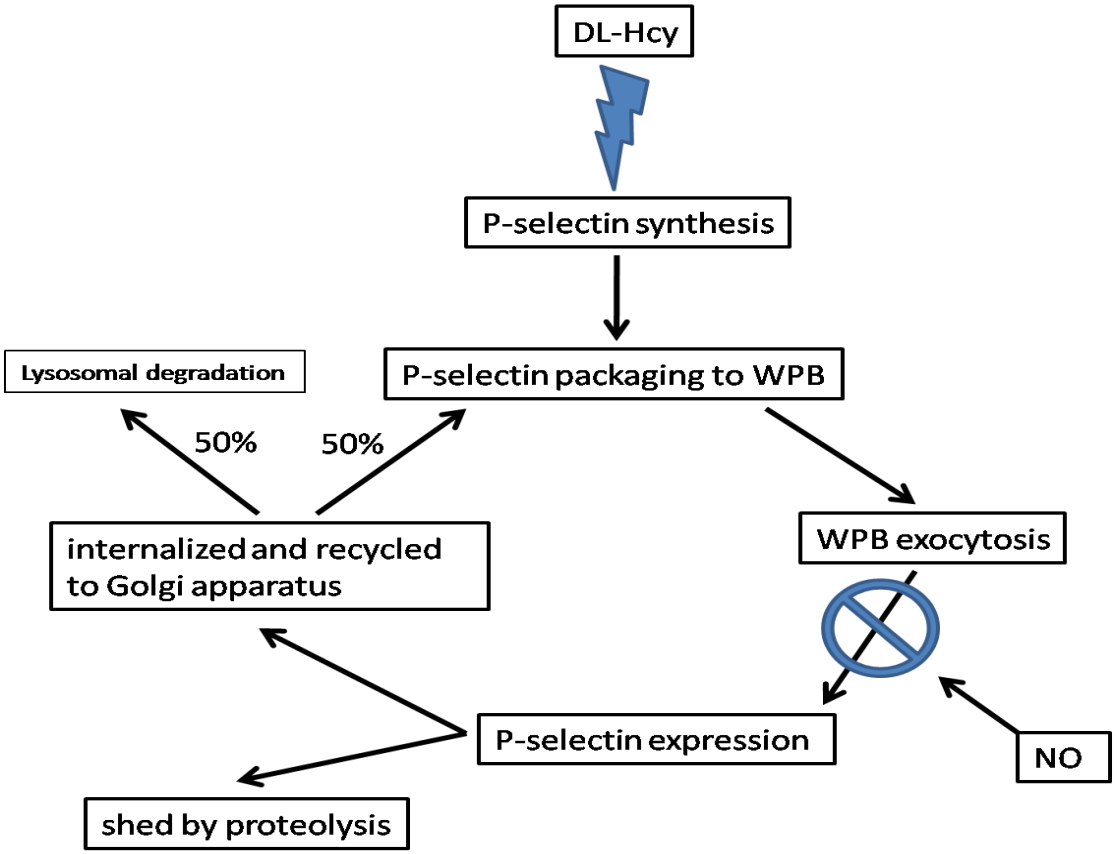


Figure 45: P-selectin life cycle and how NO can regulate P-selectin surface expression through inhibition of exocytosis.

Despite the short incubation period with NO, these results are in line with other studies which demonstrated that NO inhibits P-selectin expression (reviewed by Lowenstein (2007) and Lowenstein *et al* (2005)). Furthermore, an *in vivo* study in mice, using intravital microscopy, found that Hcy (1-5mM for 150min) induced dose- and time-dependent increases in the expression of P-selectin and ICAM-1 on venular EC which were reversed by including an NO donor (Pruefer *et al.,* 1999). NO donors have been also shown to inhibit cytokine-induced E-selectin expression in HSVEC (Zampolli *et al.*, 2000) and lipopolysaccharide-induced E-selectin expression in HUVEC (Spiecker *et al.*, 1998).

Findings using the *in vitro* flow system used in this chapter (Graham *et al.*, 2009) are in line with some other studies which used different techniques to study the effects of NO on leukocyte:EC interactions. Static adhesion assays showed that NO donors reversed leukocyte adhesion on lipopolysaccharide-treated HUVEC (Kosonen *et al.*, 2000) and on IL-1β-treated HSVEC (Zampolli *et al.*, 2000). Furthermore, intravital microscopy studies in mice showed that NO donors managed to reverse Hcy-induced (Pruefer *et al.,* 1999) and ischemia/reperfusion-induced (Gauthier *et al.*, 1994) increase in rolling and adherence of leukocytes on EC surface. Another *in vivo* study (Ahluwalia *et al.*, 2004) gave evidence that eNOS knockout mice showed increased numbers of rolling and adhered leukocytes and NO donors were able to significantly attenuate this increase and thus were consistent with this chapter's findings. They showed however that these effects happen through cGMP-dependent pathway which contradicts the mechanism suggested by this chapter which suggests that NO effects are cGMP-independent. That could be due to differences between *in vivo* and *in vitro* conditions, differences between mouse and human or NO might act via different mechanisms in different vessel types.

Nitrosylation was suggested to be a reversible process and reducing reagents like DTT can de-nitrosylate the nitrosylated Cys residues (Park *et al.*, 2006; Park *et al.*, 2000a). These authors demonstrated, in human embryonic kidney cells, that 10mM DTT reversed NO-induced JNK-1 suppression. However, this chapter found that DTT, even at much lower concentrations than those suggested in literature, affected the EC structure in a way that made it difficult to study the interactions between them and neutrophils after different sorts of treatments. Surprisingly DTT reversed, at least in part, DL-Hcy effects (Figure 42) and the only explanation we can provide is that cells are not normal anymore and possibly some of them have died. HUVEC might be highly sensitive to DTT and therefore, it was difficult to proceed with these experiments using DTT. However, using different reducing reagents which could be less harmful to HUVEC could be very beneficial to study whether NO effects depend on nitrosylation reactions or not. Detection of nitrosylation of proteins such as JNK and NSF, using techniques like the biotin switch assay or antibodies against nitrosylated proteins, would have completed the studies of the nitrosylation effects of NO. However, due to the funding and time limitation, these experiments would be recommended for future work to complete the findings of this thesis.

Under normal conditions, eNOS is the main enzyme responsible for catalyzing the generation of NO. However, absence of some cofactors like BH_4 or the substrate L-arginine induces eNOS uncoupling and it will start catalyzing $O_2^{\bullet-}$ generation at the expense of NO (Xia *et al.*, 1998). Several studies gave evidence of eNOS uncoupling as a major source of the oxidative stress in EC (Forstermann and Munzel, 2006; Landmesser *et al.*, 2003; Topal *et al.*, 2004; Zhang *et al.*, 2000). Furthermore, NADPH oxidase in EC was suggested to be activated by Hcy to start producing $O_2^{\bullet-}$ which reduces BH_4 (Zhang *et al.*, 2000) thus

uncoupling eNOS and leading to more production of $O_2^{\bullet-}$. Several studies provided evidence that NADPH oxidase in diabetic humans and mice can be a preliminary source of $O_2^{\bullet-}$ that induces eNOS uncoupling. Furthermore, inhibiting either eNOS or NADPH oxidase in human diabetic EC inhibited $O_2^{\bullet-}$ production (reviewed by Gao and Mann (2009)). Chapter 4 gave clear evidence that DL-Hcy induces oxidative stress in EC which was responsible for the DL-Hcy-mediated JNK activation and HUVEC:neutrophil interactions. However, the mechanism by which DL-Hcy is inducing ROS generation in human EC is unclear. This chapter investigated the role of eNOS in mediating DL-Hcy-induced ROS generation.

NBT reduction results showed that inhibiting eNOS reduced the generation of superoxide anions in DL-Hcy/L-NAME-treated cells compared to DL-Hcy-treated cells (Figure 43). This clearly indicates that eNOS is mediating DL-Hcy induced superoxide anion generation intracellularly under static conditions. Furthermore, inhibiting eNOS by L-NAME reversed all DL-Hcy induced HUVEC:neutrophil interactions under flow conditions to a level similar to those of non-treated cells (Figure 44) suggesting that ROS generation is the cause of EC:neutrophil interaction within this study. Furthermore, the use of second eNOS inhibitor L-NIO determined conclusively that these effects were specific to inhibiting eNOS. Using eNOS inhibitor dramatically reversed Hcy-induced oxidative stress under static and flow conditions which indicates a major role of uncoupled eNOS in mediating Hcy-induced oxidative stress. However, there might be still other sources of oxidative stress in Hcy-treated EC such as NADPH oxidase or xanthine oxidase which still need further investigation.

Different studies are in line with the results of this chapter and they illustrated that Hcy induces eNOS uncoupling. Hcy-induced ROS generation was markedly blocked by the NOS inhibitor L-NAME in HUVEC (Topal *et al.*, 2004), in BAEC (Heydrick *et al.*, 2004) and in hHcy mice (*Cbs*$^{+/-}$ mice fed the high methionine diet) (Dayal *et al.*, 2004). However, we cannot rule out as a possibility the effects of reduction of BH_4 since Topal's group found that Hcy (100µM/24h) decreased by 80% the intracellular amount of BH_4 in HUVEC leading to eNOS uncoupling (Topal *et al.*, 2004).

Collectively, it could be concluded that DL-Hcy induced eNOS uncoupling which started to produce superoxide anions in excess. These anions were responsible for the oxidative stress which in turn was responsible for initiating JNK pathway activation (as explained in Chapter 4) which ended with significant CAM expression able to mediate HUVEC:neutrophil interactions under flow conditions.

5.4. CONCLUSION

NO bioavailability is essential in protecting from CVD and Hcy induces atherosclerotic effects through affecting NO bioavailability. Therefore, increasing NO level is one possible therapeutic approach to cure and/or protect from Hcy effects. On the other hand, eNOS uncoupling can account at least in part for Hcy effects and could be the mechanism through which Hcy induces oxidative stress. Recoupling the enzyme by L-arginine or BH_4 supplement might present other possible therapeutic approaches to protect hHcy patients from CVD.

CHAPTER 6

6. THE EFFECTS OF CHRONIC EXPOSURE TO DL-HCY ON ENDOTHELIAL CELL CYTOKINE PRODUCTION

EC produce a number of cytokines and chemokines and regulate the production of many other cytokines/chemokines in an autocrine or paracrine manner (Galkina and Ley, 2009). In addition to CAM, increased production of pro-inflammatory cytokines and chemokines represent another characteristic feature of activated EC. Prolonged up-regulation of such cytokines will lead to the chronic inflammatory response. Uncontrolled and self-directed inflammatory responses might result in serious diseases such as atherosclerosis (Muller, 2002) and any factor that induces overproduction of pro-inflammatory cytokines and chemokines will favor the development of atherosclerosis.

The data from previous chapters indicate clearly the role of Hcy in activating EC and that most of Hcy effects were reproducible with acute treatment with the pro-inflammatory cytokine TNF-α (as was used as a positive control) or other pro-inflammatory mediators (Ahluwalia *et al.*, 2004; Takahashi *et al.*, 1996; Zampolli *et al.*, 2000). Furthermore, several *in vivo* studies reported a positive correlation between plasma Hcy levels and TNF-α levels in atherosclerotic patients (Abdel Aziz *et al.*, 2001; Akalin *et al.*, 2008). Collectively, if Hcy is shown to induce the synthesis of such pro-inflammatory mediators, that might explain all the reported effects of Hcy via a mechanism similar to those cytokines. Furthermore, chemokines such as IL-8 have been suggested to play a key role in activating leukocyte integrins which in turn mediate the adhesion of leukocytes on inflamed EC (Gerszten *et al.*, 1999; Rot, 1992). Therefore, this chapter will expand what is

known about the role of IL-8 and TNF-α in Hcy-induced dysfunction through detecting their production at both protein and mRNA level in chronically Hcy-treated EC.

6.1. MATERIAL AND METHODS

6.1.1. MATERIALS

Oligonucleotide primers for IL-8 forward: 5′-ATGACTTCCAA GCTGGCCGTGGCT-3′ and reverse: 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5′-ACCACAGTTCA TGCCATCAC-3′ and reverse: 5′-TCCACCACCCTGTTGCTGTA-3′ were purchased from Sigma (www.sigma.com). Agarose (15510-027) was purchased from Invitrogen (Paisley, UK) and ethidium bromide (E7637) was purchased from Sigma-Aldrich (Poole Dorset, UK). TAE buffer (H5231) was purchased from Promega, Southampton, UK. All other materials used in this chapter were provided in ready kits, for total RNA isolation: RNeasy Mini Kit (74104) and DNA digestion kit (79254) were bought from Qiagen (Crawley, West Sussex, UK); for cDNA synthesis: ImProm-II™ Reverse Transcription System (A3800) was bought from Promega, Southampton, UK.

For PCR reactions, PCR buffer (Y02028) and $MgCl_2$ (Y02016) were purchased from Invitrogen Paisley, UK. dNTP mix (U1511), Taq DNA Polymerase, (M2031) 100bp DNA ladder (G2101), loading dye (G190A) and RNase free water (P1193) were purchased from Promega, Southampton, UK. For real time PCR, Taqman gene expression mastermix (4369016) and Taqman primer/probe sets: CXCL-8 (Hs99999034_m1), TNF-alpha (Hs00174128_m1) and GAPDH (Hs99999905_m1) were all purchased from Applied Biosystems (www.AppliedBiosystems.com). Amplifications of individual genes were performed on ABI 7500 Sequence Detection System.

6.1.2. METHODS

6.1.2.1. Assessment of IL-8 and TNF-α protein production by HUVEC using ELISA

HUVEC cells were seeded into gelatin-coated 96-well plates (as explained in section 2.1.2.4) ($1x10^4$ cells/well) in triplicate and plates were incubated at 37°C and 5% CO_2 in a humidified atmosphere for 96h. Culture media was changed once after 48h and at the end of incubation time, media was collected from the wells and mixed for each triplicate. The media was centrifuged (560*g* for 15min) to pellet any cellular residue and debris. Supernatant was then collected and kept at -80°C until the ELISA experiment could be carried out.

Since DL-Hcy was shown to inhibit the growth of HUVEC (section 2.2.3.3), different numbers of cells might be present in differently treated wells and hence those wells with higher number of cells will produce a larger amount of IL-8 or TNF-α. Therefore, MTT assays were conducted on the cells after collecting the media (as described in section 2.1.2.4) and then ELISA results were adjusted according to the MTT results to eliminate any difference in IL-8 or TNF-α secretion due to differences in cell numbers.

6.1.2.1.1. Detection of IL-8 protein

A commercial kit was used to detect the IL-8 protein level in the culture media (DY208, R&D systems, Oxon, UK). The kit included all the reagents used and the assay was done as recommended by the manufacturer. Briefly, ELISA immuno 96 MicroWell™ plates (Nunc, Roskilde, Denmark) were coated with 100µl of the capture antibody (4µg/ml in PBS) and incubated overnight at room temperature. The following day, the remnants of the capture antibody were discarded and all wells were washed four times with 400µl wash buffer (0.05% v/v Tween 20 in PBS, pH 7.2 - 7.4). Plates were blotted on dry tissue after

each wash to get rid of any remaining liquid. Subsequently, 100µl blocking buffer (1% w/v BSA, in PBS with 0.05% w/v NaN_3) was added to each well and incubated for 1h at room temperature to block nonspecific binding sites. Wells were washed in the same way they were washed after the capture antibody and then 100µl of the collected media was added to each well in duplicate and incubated for 2h at room temperature. Subsequently, washing steps were repeated as before and then 100µl of the detection antibody (20ng/ml in reagent diluent: 0.1% w/v BSA, 0.05% v/v Tween 20 in Tris-buffered Saline [20 mM Trizma base, 150 mM NaCl], pH 7.2 - 7.4) was added to each well. Plates were incubated for 2h at room temperature. Following the incubation, washing steps were repeated as before and 100µl streptavidin-HRP was added after it was prepared to its working concentration (1:200) in reagent diluent. Plates were incubated for 20min at room temperature and then washed four times as before. 100µl of freshly prepared substrate solution (reagent A and B) were added to each well and incubated in dark for 20min at room temperature. The reaction with the substrate produced blue colour which was stopped by 50µl of 1M sulphuric acid. The formed colour was stable for 30min and absorbance was measured using Microplate reader MRX II (Dynex technologies, Chantilly, USA). The microplate reader was set to 450nm and then set to 540nm as a correction wavelength and then the reading produced at wavelength 540nm was subtracted from the reading produced at wavelength 450nm as recommended by the manufacturer. This subtraction will correct for the optical imperfections in the plate as readings made directly at 450nm without correction may be higher and less accurate.

Human IL-8 standard was provided with the kit as lyophilized powder which was reconstituted in dH_2O and for each assay serial dilutions of IL-8 standard were included

(2000, 1000, 500, 250, 125, 62.5 and 31.25 pg/ml). Readings of the standards were used to generate a standard curve from which the IL-8 concentrations of unknown wells were calculated using the software Revalation version 4.02.

5.1.2.1.2. Detection of TNF-α protein

A commercial kit was used to detect the TNF-α protein concentration in the culture media (DTA00C, R&D systems, Oxon, UK). The kit included all the reagents used and the assay was carried out as recommended by the manufacturer. Briefly, 50µL of assay diluent RD1F was added to each well of TNF-α Microplate (96-well plate coated with a mouse monoclonal antibody against TNF-α) and then 200µl of the collected media was added to each well in duplicate and incubated for 2h at room temperature. Subsequently, the remnants of the samples were discarded and all wells were washed four times with 400µl wash buffer using a squirt bottle. Plates were blotted on dry tissue after each wash to get rid of any remaining liquid. Following that, 200µl of HRP-conjugated polyclonal antibody against TNF-α was added to each well and incubated for 1h at room temperature. Wells were washed as previously following which, 200µl of freshly prepared substrate solution (reagent A and B) was added to each well and the plate was incubated in the dark for 20min at room temperature. The reaction with the substrate produced a blue colour and was stopped by 50µl of 1M sulphuric acid. The formed colour was stable for 30min and absorbance was measured using a Microplate reader MRX II (Dynex technologies, Chantilly, USA). The microplate reader was set to 450nm and then set to 540nm and adjustments were made for optical imperfections as described previously (section, 6.1.2.1.1.).

Human TNF-α standard was provided with the kit as a lyophilized powder which was reconstituted in dH_2O and for each assay serial dilutions of TNF-α standard were included (1000, 500, 250, 125, 62.5, 31.25 and 15.6 pg/ml). Readings of the standards were used to generate a standard curve from which the TNF-α concentrations of unknown wells were calculated using the software Revalation version 4.02.

6.1.2.2. Assessment of IL-8 and TNF-α gene expression by HUVEC

6.1.2.2.1. Extraction of RNA from cultured HUVEC

Total RNA was isolated from differently treated HUVEC using RNeasy Mini Kit. Cells were grown in T25 flasks and treated as described before with 1mM DL-Hcy, 1mM Cys or left untreated. Upon confluence, the cells were washed twice with RNase-free PBS and then cells were detached from the flask by Trypsin/EDTA (0.05%/0.02%). Afterwards, the cells were transferred into RNase-free polypropylene centrifuge tubes and centrifuged at 560*g* for 5min. Supernatant was discarded and the pellet was then washed 3-4 times by suspending in RNase-free PBS and centrifuging. The supernatant was discarded completely and the cell pellet was disrupted by adding appropriate volume of buffer RLT. The cell pellet was then loosened thoroughly by flicking the tube and mixed thoroughly by vortex ensuring that no cell clumps were visible. Cells were homogenized by passing the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) (NV-2025R, Terumo, Leuven, Belgium) fitted to an RNase-free syringe (BD Plastipak). A volume of 70% v/v alcohol (350µl) was added to the homogenized cells and mixed thoroughly by repeatedly pipetting the lysate. Afterwards, the lysate including any formed precipitates was moved to an RNeasy spin column, which contains a silica-based membrane, and centrifuged for 15s at ≥8000*g*, the flow-through was discarded and collection tube was used in the next step.

An appropriate volume of buffer RW1 (350µl) was added to the spin column, centrifuged for 15s at ≥8000*g* and the flow-through was again discarded. Reverse transcriptase PCR is highly sensitive to any traces of DNA so at this stage a DNA digestion step was performed to eliminate any possibilities of contamination with DNA using RNase-Free DNase set. The lyophilized enzyme DNase I was dissolved in 550µl RNase-free water (final concentration: 2.73Kunitz units/ml), mixed gently and then aliquoted and stored at -20°C. DNase I was diluted (1:8) in the buffer RDD and then 80µl of the diluted enzyme were added to the spin column membrane, and placed at room temperature for 15min. At the end of the incubation, an appropriate volume of buffer RW1 (350µl) was added to the spin column, centrifuged for 15s at ≥8000*g*, the flow-through was discarded and subsequently, 500µl of buffer RPE were added to the RNeasy spin column and centrifuged for 15s at ≥8000*g*, the flow-through was discarded and washing with 500µl buffer RPE was repeated again but with centrifuging for 2min at ≥8000*g*. The long centrifugation dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution. To eliminate any possible carry over of Buffer RPE, the spin column was placed on new 2ml collection tube and centrifuged at full speed for 1min. RNeasy spin column was then placed in a new 1.5ml collection tube and 40µl of RNase-free water were added directly to the spin column membrane and centrifuged for 1min at ≥8000*g* to elute the RNA. The last step was repeated with another volume of RNase-free water (40µl). 40µl of the isolated RNA were used in the later step for quantifying and determining the quality of the isolated RNA and the rest was kept in -80°C.

6.1.2.2.2. Quantification and determination of quality of RNA

Spectrophotometry

The quantity of RNA in each sample was checked using spectrophotometry. RNA was diluted in RNase-free water (1:20) and then the absorbance of UV was detected using a Jenway 6305 UV/vis spectrophotometer (Keison products, Essex, UK) at two different wavelengths 280nm for DNA and 260nm for RNA. The spectrophotometer was first calibrated to zero using nuclease-free water in a quartz cuvette. RNA purity was evaluated by calculating the ratio (absorbance at 260nm/absorbance at 280nm) and RNA quality was considered good if the ratio was between (1.8-2.2). RNA concentration was determined using the equation where Ab260 is the absorbance at 260nm and DF is the dilution factor:

$$\text{RNA concentration } (\mu g/ml) = Ab260 \times DF \times 40$$

Agarose gel electrophoresis

A 1.5% gel was formed by melting agarose in 1X tris-acetate-EDTA buffer (TAE) using a 950W microwave (Proline Microchef ST44) on power 7 for 135s till the agarose was completely dissolved. Subsequently, the solution was left at room temperature to cool down to approximately 50°C and then ethidium bromide was added to the solution and mixed (final concentration of 0.6µM). The solution was poured into plastic gel trays containing gel combs to form wells, and left for approximately 45min to set. Subsequently, gel combs were removed gently and the gel was placed in an electrophoresis tank containing enough 1x TAE buffer with ethidium bromide (0.6µM) to cover the gel. 15µl of RNA samples were mixed with 3µl of loading dye in a ratio of 5:1 to assist loading and monitoring progression of the total RNA through the gel throughout electrophoresis, and

then carefully loaded into the gel. The tank was set to run at 100V for approximately 30min. Subsequently, the UVitec gel documentation system (UVItec Limited, Cambridge, UK) was used to visualise total RNA and the images were captured using software Uvitec version 12.06 for windows.

6.1.2.2.3. Complementary DNA (cDNA) synthesis

The RNA purified in section 6.1.2.2.1 was reverse transcribed to complementary DNA which in turn is ideal for PCR experiments. ImProm-II™ Reverse Transcription System was used. The protocol recommended by the manufacturer was followed. Briefly, all reagents and RNA samples were thawed on ice and then any unused portion returned to the freezer as soon as aliquots were taken. Reaction mix was prepared in 0.5ml eppendorf tubes (Alpha Laboratories) on ice and included ImProm-II™ 5X reaction buffer (16µl), 9.6µl $MgCl_2$ (6mM), 4µl dNTP Mix (10mM of each dNTP), 2µl oligo(dT)15 primer (1µM), 4µl ImProm-II™ reverse transcriptase, 18µl RNA samples (up to 2µg/reaction) and then volume was made up to 80µl by Nuclease-Free Water (26.4µl). RNA and all reagents were mixed thoroughly by carefully vortexing for no more than 5s and were centrifuged briefly to ensure all the liquid was collected at the bottom of the tube. Subsequently, tubes were placed in the PCR Sprint thermal cycler (Thermo Hybaid, Ashford, UK) and set to run the reverse transcription programme which was: incubation at 25°C for 5min for annealing followed by incubation at 42°C for up to one hour to allow cDNA synthesis from mRNA by reverse transcription. Afterwards, the reactions were stopped and reverse transcriptase was inactivated at 70°C for 15min. Produced cDNA was aliquoted and maintained at -20°C for long-term storage.

6.1.2.2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

All reagents were thawed on ice and then, any unused portion returned to the freezer as soon as aliquots were taken. PCR reaction mix was prepared in 0.5ml Eppendorf tubes (Alpha Laboratories) on ice by mixing all of the following reagents: 5µl PCR buffer, 2.5µl $MgCl_2$ (3.125mM), 1µl dNTP mix (10mM each), 0.5µl Taq DNA Polymerase (5u/µl), 2.5µl forward primer (10µM, IL-8 or GAPDH), 2.5µl reverse primer (10µM, IL-8 or GAPDH) and then the volume was made up to 40µl by 26µl RNase free water. After preparing the reaction mix, 10µl of the cDNA samples was mixed with 40µl of master reaction mix by gently pipetting in PCR tubes. 40µl of the master reaction mix was mixed with 10µl of RNase-free water instead of cDNA and this served as a negative control to check if there was any DNA contamination. cDNA samples with all reagents were mixed thoroughly by carefully vortexing for no more than 5s, and centrifuged briefly to ensure all the liquid was collected at the bottom of the tube. Subsequently, PCR was performed in PCR Sprint thermal cycler (Thermo Hybaid, Ashford, UK) and PCR conditions for IL-8 were: initial denaturation at 94°C for 5min and then (denaturation at 94°C for 30s, annealing at 65°C for 30s and extension at 68°C for 1min) for 28 cycles with a final extension at 68°C for 2 min. PCR conditions for GAPDH were: initial denaturation at 95°C for 15min and then (denaturation at 94°C for 1min, annealing at 63°C for 1min and extension at 72°C for 1min) for 25 cycles with a final extension at 72°C for 10min. The PCR products were placed on ice to be analysed immediately by agarose gel electrophoresis as explained in section 6.1.2.2.2. A DNA ladder was loaded in the gel and used as a molecular size marker. The PCR products were electrophoresed on a 1.5% w/v TAE-agarose gel and visualized by ethidium bromide staining. The PCR product size for

amplified IL-8 and GAPDH were 267bp and 450bp respectively. UVitec gel documentation system (UVItec Limited, Cambridge, UK) was used to visualise the PCR products and the images were captured using software Uvitec version 12.06 for windows. The differences in IL-8 expression were estimated by measuring the intensity of the bands using ImageJ software and values were normalized against GAPDH bands density for each sample.

6.1.2.2.5. Real time PCR

For quantitative PCR, the following mix was prepared (Taqman Universal PCR Master Mix: 10µl, Taqman Primer/Probe: 0.5µl, cDNA: 2µl and H_2O: 7.5µl) and 20µl of this mix was distributed in MicroAmp® optical reaction 96-well plate (4306737, Applied Biosystems). Samples were prepared in triplicate and the plates were centrifuged briefly to ensure a good mix of reagents. Real-time PCR was performed using the Applied Biosystems 7500 Real-Time PCR System. The thermocycler parameters were 50°C for 2min and 95°C for 10min, followed by 50 cycles of 95°C for 15s and 60°C for 30s. The threshold cycle (C_T), which indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold, from each well was determined using the Applied Biosystems Sequence Detection Software v2.01. Relative quantification representing the change in gene expression from real-time quantitative polymerase chain reactions between experimental groups was calculated by the comparative C_T method. The data was analyzed by calculating the relative quantification (RQ) using the equation: RQ = $2^{-\Delta CT}$x100, where $\Delta C_T = C_T$ of target gene$-C_T$ of housekeeping gene. Evaluation of $2^{-\Delta CT}$ indicates the fold change in gene expression, normalized to the internal control (GAPDH) which enabled the comparison between differently treated cells. All results then were

logged to the base 10 ($Log_{10}x$) and a ratio paired t-test was applied to detect the significant level of the differences between differently treated cells.

6.2. RESULTS

6.2.1. The effects of DL-Hcy on cytokine protein secretion

6.2.1.1. IL-8

The secretion of IL-8 in response to DL-Hcy treatment was detected using ELISA and concentrations of IL-8 protein in culture media were adjusted according to the number of live cells (assessed by MTT) in each well. Non-treated cells showed basal detectable concentrations of IL-8 in the culture media. However, DL-Hcy-treated HUVEC expressed a higher concentrations of IL-8 protein (1910 $\pm$ 65 pg/ml) compared to non-treated cells (1613 $\pm$ 39 pg/ml) (p<0.01). Figure 46 represents results from 6 individual experiments using 6 different donors. In contrast, cells treated with Cys showed concentrations of IL-8 protein (1475 $\pm$ 122 pg/ml) similar to non-treated cells.

5.2.1.2. TNF-α

The secretion of TNF-α in response to DL-Hcy treatment was detected using ELISA and concentrations of TNF-α protein in culture media were adjusted according to the number of live cells (assessed by MTT) in each well. TNF-α protein was not detectable in the culture media of any of the treated or non-treated cells.

The effects of chronic exposure to DL-Hcy on IL-8 protein production

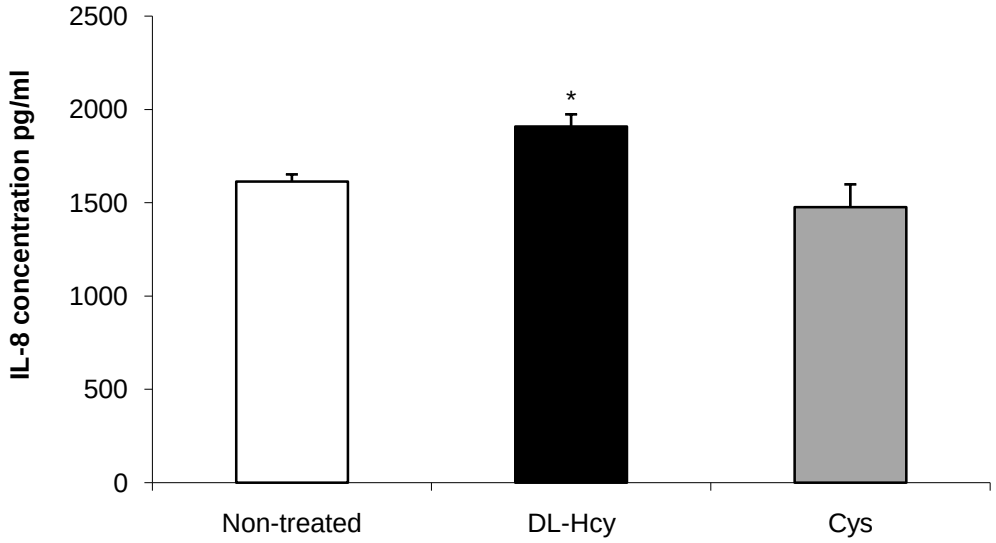


Figure 46: HUVEC were grown in complete M199 medium with DL-Hcy (1mM; 5-9 days), or Cys (1mM; 5-9 days). HUVEC from the same donor were grown in complete M199 media without any stimulation and considered with Cys-treated cells as negative controls. IL-8 protein production was detected in the culture media using ELISA and results were adjusted to the numbers of live cells in each well which were estimated using MTT. The results are expressed as mean (+/- SEM) from 6 different donors for DL-Hcy, Cys and non-treated (n=6). (*) $p < 0.01$ for DL-Hcy versus non-treated cells.

6.2.2. The effects of DL-Hcy on cytokine mRNA expression

6.2.2.1. RNA isolation efficiency

Total RNA was isolated from HUVEC as described in section 6.1.2.2.1 and the efficiency of the purification method was assessed by running the isolated RNA on 1.5% agarose gel (Figure 47). The image in Figure 47 shows RNA isolated from three different samples, non-treated, DL-Hcy-treated and Cys-treated HUVEC. Two main bands for each sample could be identified representing the two ribosomal RNA (rRNA) components 18S and 28S. 28S rRNA band was approximately twice as intense as the 18S rRNA band. This indicates that isolated RNA was of a good quality as intact RNA has these two bands with a 2:1 ratio (28S:18S). On the other hand, degraded RNA will not exhibit 2:1 ratio and will appear as a very low molecular weight smear (Skrypina *et al.*, 2003). This experiment was repeated after each isolation process and the image displayed in Figure 47 represents results of 4 different experiments.

6.2.2.2. Reverse transcriptase PCR

The expression of IL-8 mRNA was assessed in differently treated cells using reverse transcriptase PCR. GAPDH was used as an endogenous internal control for variations in PCR product levels due to any differences in RNA isolation efficiency and PCR reaction efficiency. Furthermore, variations in the numbers of cells between differently treated flasks due to inhibitory effects of DL-Hcy on cell proliferation were adjusted by normalizing the results of each sample against its own level of GAPDH as GAPDH is expressed constitutively and not affected by DL-Hcy. Having loaded equal amounts of RNA in the PCR reaction, it can be concluded that GAPDH is expressed constitutively and is not affected by DL-Hcy treatment (Figure 48).

All cells produced PCR products which correspond to the expected amplicon size (267bp: IL-8 and 450bp: GAPDH) and Figure 48A shows bands of IL-8 and GAPDH RNA from one experiment. There were no bands in the negative control in which the cDNA was replaced with nuclease-free water, indicating that the reagents used in the PCR reaction mix were free from any contamination. All IL-8 RNA bands had the same density in differently treated cells and that was confirmed by quantifying the bands using ImageJ software (Figure 48B). Results indicate no changes in IL-8 mRNA expression in HUVEC once these cells were treated with DL-Hcy compared to non-treated or Cys-treated cells.

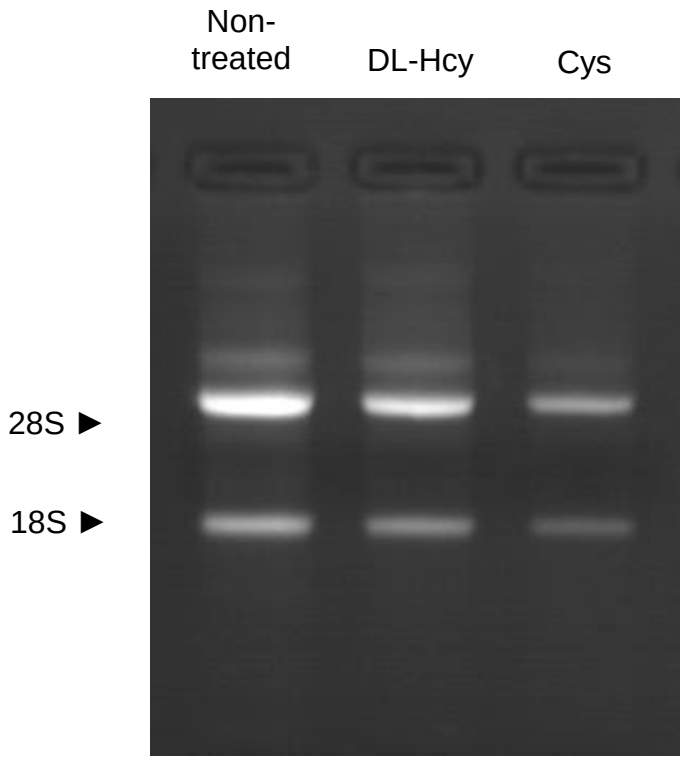


Figure 47: Representative image showing the efficiency of RNA isolation. 15µl of purified total RNA was loaded on 1.5% TAE-agarose gel, separated by electrophoresis and visualized by UVitec gel documentation system. The 28S and 18S ribosomal RNA are clear in the total RNA samples with a ratio of approximately 2:1.

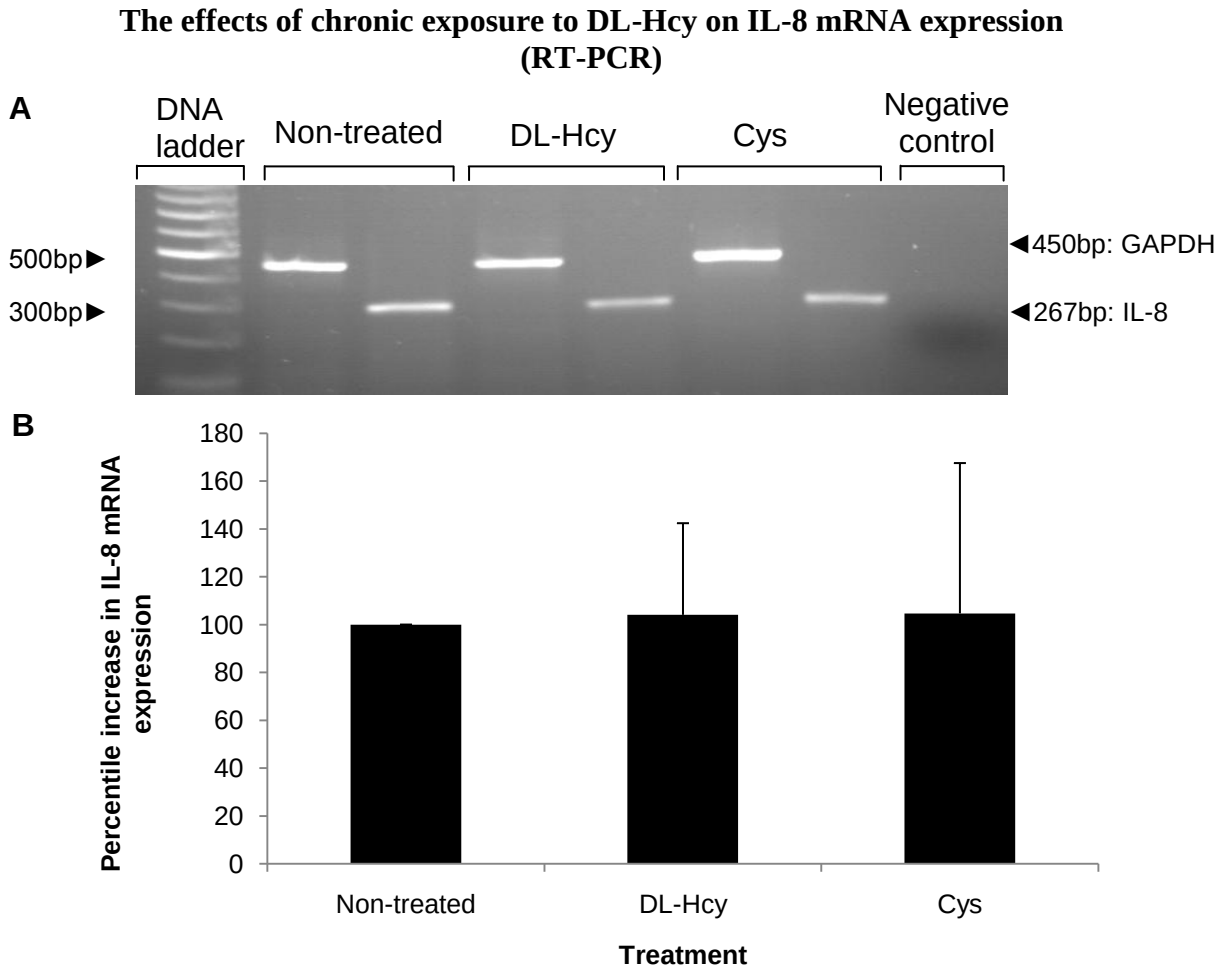


Figure 48: A: Representative TAE-agarose gel photographs showing ethidium bromide stained RT-PCR products of IL-8 mRNA. HUVEC were grown in complete M199 medium with DL-Hcy (1mM; 5-9 days), or Cys (1mM; 5-9 days). HUVEC from the same donor were grown in complete M199 media without any stimulation and considered with Cys-treated cells as negative controls. IL-8 and GAPDH mRNA from differently treated cells were amplified using RT-PCR as described in section 6.2.2. No bands were observed in the negative control. The expected amplicon size is 267bp and 450bp for IL-8 and GAPDH respectively. B: Density of the bands represented in Figure 48A and from 2 further experiments with independent samples were quantified using ImageJ software. Non-treated values were defined as 100% and other values were adjusted accordingly. Results are expressed as mean (+/- SD) from 3 different donors (n=3).

6.2.2.3. Real time PCR

Quantitative real time PCR was applied to study the expression of the mRNA of IL-8 RNA and TNF-α and all results were adjusted to the internal endogenous control GAPDH. Figure 49 shows the numbers of PCR amplifying cycles required for each gene to reach a fixed threshold.

To confirm the findings of RT-PCR for IL-8, a quantitative real time PCR was applied and the results show that DL-Hcy induced a slight increase in the level of IL-8 mRNA in DL-Hcy-treated cells compared to non-treated ones. However, this increase was not significant even when a ratio paired t-test was applied (Figure 50). On the other hand, DL-Hcy-treated cells showed about 5-fold increase in their TNF-α mRNA. Ratio paired t-test was applied and indicated that the increase in TNF-α mRNA was significant ($p<0.05$) (Figure 51).

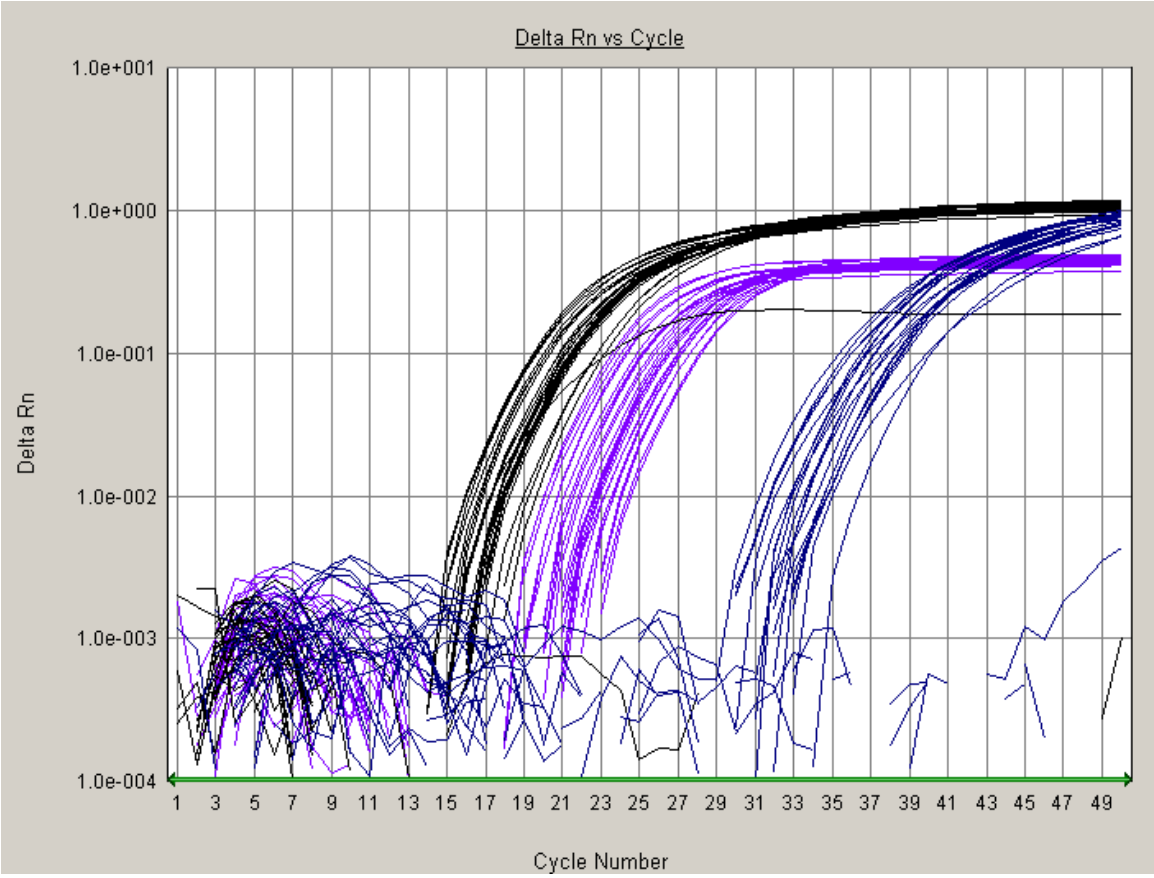


Figure 49: The number of PCR cycles required for each gene to reach a fixed threshold. The genes which were amplified are: GAPDH (black), IL-8 (violet) and TNF-α (blue).

The effects of chronic exposure to DL-Hcy on IL-8 mRNA expression (real time-PCR)

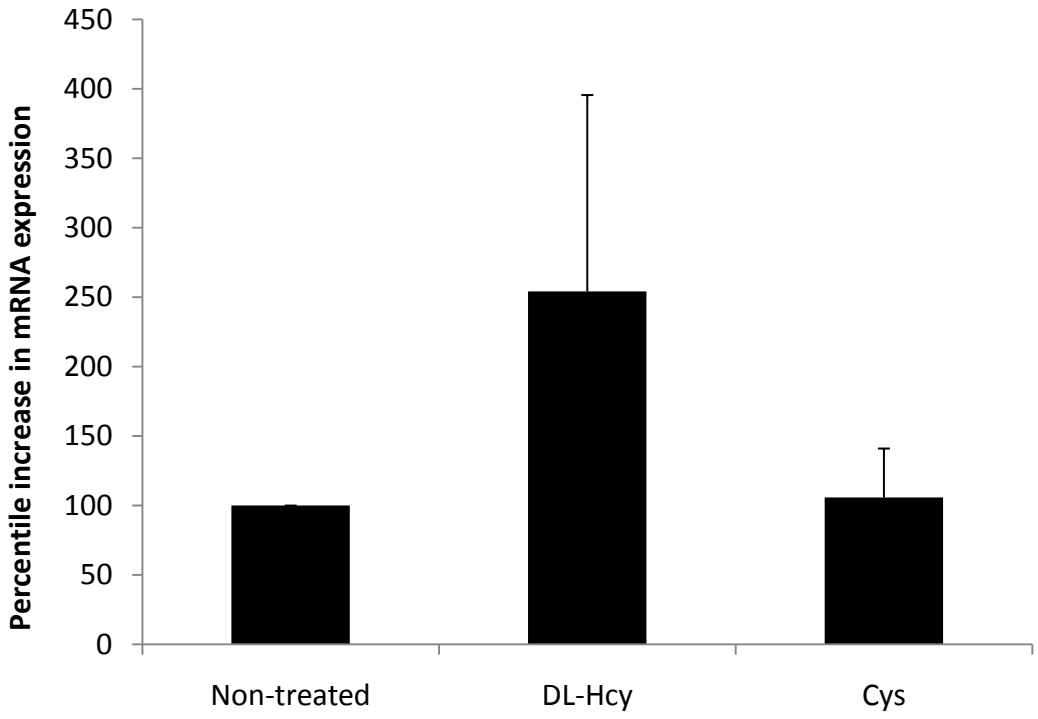


Figure 50: HUVEC were grown in complete M199 medium with DL-Hcy (1mM; 5-9 days), or Cys (1mM; 5-9 days). HUVEC from the same donor were grown in complete M199 media without any stimulation and considered with Cys-treated cells as negative controls. IL-8 mRNA was amplified in differently treated cells using real time PCR. Non-treated values were defined as 100% and other values were adjusted accordingly. Results are expressed as mean (+/- SEM) from 4 different donors for DL-Hcy, Cys and non-treated (n= 4).

The effects of chronic exposure to DL-Hcy on TNF-α mRNA expression (real time-PCR)

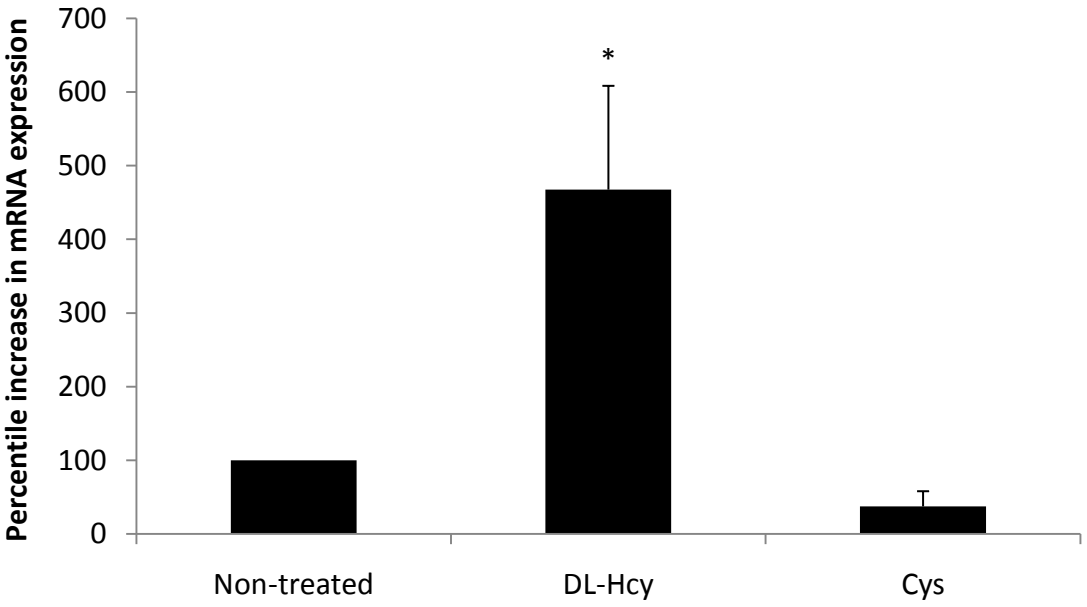


Figure 51: HUVEC were grown in complete M199 medium with DL-Hcy (1mM; 5-9 days), or Cys (1mM; 5-9 days). HUVEC from the same donor were grown in complete M199 media without any stimulation and considered with Cys-treated cells as negative controls. TNF-α mRNA was amplified in differently treated cells using real time PCR. Non-treated values were defined as 100% and other values were adjusted accordingly. Results are expressed as mean (+/- SEM) from 4 different donors for DL-Hcy, Cys and non-treated (n= 4). (*) $p < 0.05$ for DL-Hcy versus non-treated cells.

6.3. DISCUSSION

The role of chemokines in mediating the pathology of atherosclerosis was addressed in several studies reviewed by Tedgui and Mallat (2006), and different groups have previously investigated whether acute administration of Hcy induces synthesis of such chemokines (Dalal *et al.*, 2003; Poddar *et al.*, 2001; Zeng *et al.*, 2003). However, none of these studies considered whether chronic treatment with Hcy might induce sustained expression of such chemokines in EC. Sustained expression of pro-inflammatory cytokines and chemokines might represent another mechanism which is involved at least in part in mediating Hcy proatherosclerotic effects. Therefore, this chapter focuses on one of the major chemokines, IL-8, and its response to chronic treatment with DL-Hcy. Furthermore, the ability of EC to produce the key pro-inflammatory cytokine, TNF-α, was investigated in this chapter in addition to studying the effects of chronic treatment with DL-Hcy on such production.

All of the cell populations studied expressed detectable levels of IL-8 protein in their culture media which indicates a constitutive production of IL-8 in resting HUVEC. Cells which were treated chronically with DL-Hcy expressed significantly higher concentrations of IL-8 protein in their culture media compared to non-treated and to Cys-treated cells ($p<0.01$) (Figure 46). However, amplifying IL-8 mRNA using RT-PCR showed no significant difference in its level between the differently treated cells (Figure 48) and to confirm this finding, quantifiable real time PCR was conducted on the same samples and showed that DL-Hcy induced an increase in the mRNA expression compared to non-treated. However, this increase was variable and did not reach significance (Figure 50). Collectively, although DL-Hcy did not have measurable effects on IL-8 gene at the

transcriptional level, it did induce a small increase in IL-8 synthesis and release into the culture media. Different possibilities might account for these results; mRNA is produced in both the presence and absence of DL-Hcy as it was detected by PCR in all samples. However, DL-Hcy might activate the translation of the IL-8 mRNA and/or inhibit protein turn over and both possibilities would result in the same consequence, production of more IL-8 protein. The increase in P-selectin expression in response to chronic DL-Hcy treatment, which has been shown in Chapter 2, indicates increase in WPB exocytosis as it is the main mechanism for P-selectin to be mobilized to the cell surface. Furthermore, DL-Hcy might induce WPB exocytosis by decreasing NO level, which is suggested to be a negative regulator of WPB exocytosis (as explained in Chapter 5). Meanwhile, IL-8 is stored in WPB (Wolff *et al.*, 1998) which means the DL-Hcy-induced WPB exocytosis will result in IL-8 being mobilized outside the cells leading to increase in IL-8 protein in culture media. Collectively, that might represent a third possibility indicating that IL-8 synthesis rate is not affected by DL-Hcy however, DL-Hcy enhances its transport to WPB and the WPB exocytosis leading to releasing more IL-8 extracellularly. Furthermore, only 2% of the produced IL-8 has previously been shown to present on the EC surface, while the remainder will be shed and present in a soluble form (Marshall *et al.*, 2003). Marshall's group illustrated that shedding of IL-8 and release into culture media is mediated by tPA activity and this process was suggested as a mechanism to stabilize the IL-8 expression at the cell surface. Marshall's group suggested that soluble IL-8 has anti-inflammatory effects in contrast to the proinflammatory effects of cell surface-bound IL-8. Since tPA was suggested to be inhibited by Hcy (Hajjar *et al.*, 1998), there will be a decrease in shedding IL-8 and therefore less production of soluble IL-8 in response to Hcy treatment. However,

this chapter's results indicate a Hcy-mediated increase in soluble IL-8 despite the expected decrease due to tPA inhibition. Therefore, the small increase in soluble IL-8 is likely to reflect a more significant increase of IL-8 *de novo* synthesis and mobilization to the cell surface where only a small fraction will be shed. Therefore, our data suggest a large increase in cell surface IL-8 which would be able to promote EC activation. ELISA could be used to detect the IL-8 expression on the cell surface of Hcy-treated EC.

ROS has been shown to differentially mediate IL-8 mRNA expression and protein production in different EC and epithelial cells (Lakshminarayanan *et al.*, 1997). The same group demonstrated that hydrogen peroxide was able to induce an increase in IL-8 gene expression in human epithelial cells but not in a human microvessel endothelial cell line. Furthermore, they showed that hydrogen peroxide induced IL-8 protein secretion in both human epithelial cells and human microvessel endothelial cell line but not in HUVEC. Therefore, ROS involvement in mediating IL-8 production varies between different cell types. In contrast to Lakshminarayanan's findings, Kerkeni *et al* (2006) found that antioxidants were able to reverse, at least in part, Hcy-induced-IL-8 production in HUVEC which suggests a role for ROS in mediating IL-8 production in HUVEC. The data of Kerkeni's group might suggest that ROS works synergistically with the above suggested mechanisms to mediate the chronic DL-Hcy-induced IL-8 production showed in this chapter.

The constitutive production of IL-8 in resting HUVEC presented in this chapter matches with other studies that also reported constitutive expression of IL-8 in HUVEC (Geisel *et al.*, 2003; Lakshminarayanan *et al.*, 1997; Marshall *et al.*, 2003), HAEC (Poddar *et al.*, 2001) and HSVEC (Dalal *et al.*, 2003). Furthermore, the results of this chapter are in

line with other studies who found that Hcy induces increase in IL-8 protein synthesis in HAEC (50μM for up to 8h) (Poddar *et al.,* 2001) and in HUVEC (1-10mM, 3-24h) (Geisel *et al.,* 2003; Kerkeni *et al.,* 2006). On the other hand, Dalal *et al* (2003) failed to report increase in IL-8 production in response to Hcy (0.5-5mM for 4-24h) in HSVEC but even found that Hcy inhibited IL-8 production in dose dependent manner. The difference between this chapter's results and Dalal's group results could be due to different responses by different EC types or the short incubation time, which might have induced transient expression which then dropped back to basal levels. Furthermore, pre-treatment of cytokine-treated human VSMC with Hcy (up to 1mM for 4h) increased cytokine-mediated up-regulation of IL-8 protein and mRNA (Desai *et al.,* 2001), while other groups found that Hcy alone induced IL-8 mRNA expression in HUVEC (Geisel *et al.,* 2003) and monocytes (Zeng *et al.,* 2003). In Desai's study, Hcy might have worked synergistically with cytokines to induce the increase in mRNA level of IL-8 which might be the reason for the contrast to our results where Hcy alone was not able to induce IL-8 mRNA expression. Geisel's group incubated EC with Hcy for 20h which might have induced temporarily increase in mRNA which has then dropped back to the basal level as measured in this chapter's results. On the other hand, Zeng's study was conducted on monocytes which might respond differently to Hcy treatment from EC and that could explain why IL-8 mRNA was increased in response to Hcy alone.

JNK was shown to be involved in mediating cytokine-induced IL-8 up-regulation in cancer cells while p38 was not involved in this up-regulation (Krause *et al.,* 1998). Furthermore, the IL-8 promotor has been shown to have binding sites for AP-1, the downstream transcription factor of JNK, and binding of AP-1 is required for inducing

transcription activity of the L-8 promotor (Wu *et al.*, 1997). In contrast, Zeng *et al* (2003) demonstrated that Hcy induces IL-8 and MCP-1 production in monocytes in a p38 and ERK dependent way. This group also illustrated that the Hcy-induced production of these cytokines is ROS mediated. Therefore, it could be concluded that the pathway through which IL-8 synthesis is up-regulated varies between different cell types and between different stimuli.

The effect of Hcy on IL-8 protein production is specific to DL-Hcy since Cys was not able to induce significant increase in IL-8 protein level (Figure 46). This agrees with Poddar's group who showed in HAEC that induction of IL-8 and MCP-1 expression is specific to Hcy and none of the other thiol containing amino acids, like Cys or methionine was able to induce such expression (Poddar *et al.*, 2001). On the other hand, the same group found that DL-Hcy induced mRNA expression of MCP-1 and IL-8 in HAEC with maximal effects at 50μM and IL-8 mRNA remained high at all concentrations above 50μM (up to 10mM). This concentration was sufficient to induce increases in protein secretion within 15min of administration. Differences between cell types might account for the difference between this chapter's results and Poddar's group results concerning mRNA expression. They used arterial cells which represent a good model for atherosclerotic lesions and those cells might be more sensitive to Hcy treatment on the transcriptional level than the cells used in this chapter (HUVEC).

The second part of this chapter focuses on the TNF-α as a possible mediator for Hcy atherogenic effects. The data from real time PCR experiments revealed that HUVEC chronically treated with DL-Hcy expressed 5-fold increase in their TNF-α mRNA compared to non-treated HUVEC (Figure 51). In spite of this dramatic increase in TNF-α

mRNA, a paired t-test showed that this increase was insignificant. The raw data show that the differences between the values of non-treated and DL-Hcy treated cells are not very consistent. Thus, the ratio paired t-test was used as an alternative to t-test since it studies the differences between the ratios of the non-treated to treated values instead of studying the differences between the mean values. These ratios are much more consistent, and therefore, analyzing their logs can give more persuasive results. The ratio paired t-test showed that the increase in TNF-α mRNA noticed in DL-Hcy treated cells was significant compared to non-treated cells ($p<0.05$). Therefore, in contrast to the lack of increases observed in IL-8 mRNA, DL-Hcy induced transcription of TNF-α gene. On the other hand, none of the cell populations studied expressed any detectable concentrations of TNF-α in their culture media. Collectively, although DL-Hcy induced increase in the transcription of TNF-α gene, it could not induce increase in the synthesis rate of TNF-α protein.

These results are hard to explain and the literature does not support the ability of EC to produce TNF-α (Dalal *et al.*, 2003; Kerkeni *et al.*, 2006; Nikitina *et al.*, 1997). Dalal's group found that neither resting nor Hcy-treated HSVEC expressed detectable levels of TNF-α (Dalal *et al.*, 2003); and Kerkeni's group found also that Hcy (1-10mM, up to 24h) was not able to induce TNF-α secretion in HUVEC (Kerkeni *et al.*, 2006). On the other hand, one *in vitro* study (Bai *et al.*, 2007) showed some controversy in their findings as they found that HUVEC expressed a constitutive production of TNF-α (less than 100pg/ml) and that 100μM Hcy induced 10-fold increase in this production. ELISA used in this thesis detected levels of TNF-α as low as 15.6pg/ml so it should have been able to pick up equivalent concentrations of TNF-α to those suggested in Bai's study. This group used HUVEC at passage number between 4-6 while in this chapter HUVEC were used at

passage 2-4. Therefore, the cells in Bai's study were grown in culture conditions for longer time than those used in this chapter and that could have altered their sensitivity to stimuli or changed constitutive gene expression which could explain their production of TNF-α. Furthermore, Bai's group didn't carry out any characterization of the HUVEC they used, such as detecting the constitutive expression of vWF or CD31. This might suggest that those HUVEC are no longer normal at such high passage number, which might explain their constitutive production of TNF-α and their sensitivity toward Hcy treatment.

The increase in mRNA expression in this chapter's experiments could be explained by a low stability of the mRNA transcript, and thus, this synthesized mRNA was not translated into protein. One study found that up to 10mM Hcy was not able to induce expression of TNF-α mRNA in HUAEC (Poddar *et al.*, 2001) which contradicts the findings of this chapter. However, they used a northern blot technique to identify the expression of TNF-α mRNA and could not detect any expression in neither non-treated nor Hcy-treated cells; while in this chapter real time-PCR has been used to detect the expression of TNF-α mRNA and this technique is quantifiable and more sensitive than the technique used in Poddar's study. Furthermore, they used HUAEC in their study which might respond differently to Hcy than HUVEC does.

Surprisingly, some studies found that 5mM Hcy has inhibitory effects on TNF-α-induced up-regulation of CAM (Roth *et al.*, 2001; Stangl *et al.*, 2001), and chemokines (Roth *et al.*, 2001) in HUVEC. Their findings contrast with Desai's group who found that 1mM Hcy augmented TNF-α-induced chemokines expression in human VSMC (Desai *et al.*, 2001). The high Hcy concentration which Roth's group has used might have some inhibitory effects on the HUVEC and altered their response to different stimuli and

therefore their response to TNF-α was affected. Other cell types have been shown to respond differently to Hcy as an *in vitro* study in human monocytes (Su *et al.*, 2005) showed that DL-Hcy (185μM-720μM, 3h) induced significant increases in the mRNA expression of several different proinflammatory cytokines, TNF-α, IL-1β, IL-6, IL-8 and IL-12. Furthermore, all of these cytokine protein concentrations increased after DL-Hcy treatment (185μM-2160μM, 3h) indicating that the translation was induced by this treatment.

Very few *in vivo* studies have addressed the correlation between Hcy and TNF-α concentrations. Diabetic patients who have atherosclerotic lesions were shown to have elevated concentrations of both TNF-α and Hcy with a significant positive correlation between the two markers (Abdel Aziz *et al.*, 2001; Akalin *et al.*, 2008). Furthermore, patients with vitamin B12 deficiency showed significant increase in Hcy and TNF-α concentrations and their concentrations were significantly correlated (Peracchi *et al.*, 2001). Vitamin B12 is an essential cofactor for remethylation of Hcy which explains the high concentrations of Hcy in those patients. However, it remains unclear whether the increase of TNF-α is due to vitamin B12 being involved in regulating TNF-α synthesis or due to direct induction of TNF-α synthesis by accumulated Hcy. Interestingly, a study on patients with psoriatic arthritis found that blocking TNF-α function led to a significant decrease in Lp(a) and Hcy (Sattar *et al.*, 2007). Sattar's findings might suggest a possible role of TNF-α in regulating Hcy metabolism while other *in vitro* studies (Bai *et al.*, 2007; Su *et al.*, 2005) suggest a role for Hcy in inducing TNF-α synthesis. Therefore, the actual relation between the atherogenic Hcy and the proinflammatory cytokine TNF-α is still controversial

and needs more investigation to reveal their effects on each other's production and their effects on each other's efficiency.

To sum up, there are some evidence that a positive correlation between Hcy and TNF-α is present in atherosclerotic patients or those at risk of developing atherosclerosis, and both Hcy and TNF-α might play a key role in atherosclerosis progression. However, this observed correlation was recorded in patients. Hence, different cells could participate in TNF-α production. Furthermore, it is not clear whether EC produces TNF-α and whether this production could be induced by Hcy. Despite the findings of this chapter that Hcy induced TNF-α mRNA expression, the protein level of TNF-α was undetectable and therefore, these results are hard to explain and are not final. More work is still needed to explore the role of TNF-α in Hcy-induced EC damage. Furthermore, there is a need to find a positive control that can induce TNF-α production by EC in order to confirm that EC are able to produce TNF-α. It might be worth trying the use of lipopolysaccharides, TNF-α itself or other proinflammatory cytokines like IL-1 as an inducer of TNF-α production. Furthermore, IL-1 has been shown to be produced by EC (Libby *et al.*, 1986) and therefore, it could present a possible mediator of Hcy atherogenic effects. Therefore, characterizing the expression of IL-1 mRNA and its protein production could be a possible future step to characterize the role of pro-inflammatory cytokines in mediating Hcy-induced EC activation.

6.4. CONCLUSION

This chapter provide strong evidence that DL-Hcy affects the production of the chemokine IL-8 which is thought to play a key role in mediating DL-Hcy-induced EC activation. These activated EC will express several CAM on their surface which work

together with the secreted chemokines to recruit leukocytes and start the inflammatory process. Even though, DL-Hcy induced TNF-α mRNA expression it did not affect its protein synthesis. The significance of this finding is therefore not clear.

CHAPTER 7

7. CONCLUSION AND FUTURE STUDIES

7.1. Conclusion

The theory of Hcy and atherosclerosis was formulated in the middle of 1970s (McCully and Wilson, 1975) and then Hcy was suggested as a primary risk factor in cardiovascular diseases by Lentz *et al* (Lentz *et al.*, 1996). Many studies have been conducted to investigate Hcy-mediated atherogenic effects and to study the mechanisms behind such effects. Most of these studies have investigated the effects of acute treatment with Hcy and also most of them studied Hcy effects under static conditions (Dong *et al.*, 2005; Postea *et al.*, 2006; Silverman *et al.*, 2002; Stuhlinger *et al.*, 2001). However, acute stimulation does not reflect a clinically relevant situation since the EC in patients with hHcy will be continuously exposed to high levels of Hcy, which may modulate the EC behaviour. Therefore, the current study aimed to characterize the effects of chronic treatment with Hcy on EC under both static and flow conditions thus making the study more pathologically relevant.

In all of the experiments carried out in this thesis, DL-Hcy up to 1mM did not kill EC. However, DL-Hcy did suppress EC proliferation rate and induced differential gene expression which led to sustained up-regulation of a number of endothelial CAM: P-selectin, E-selectin and ICAM-1. DL-Hcy also induced EC secretion of the proinflammatory chemokine IL-8. The expression of E-selectin and ICAM-1 was detected under static and flow conditions, while P-selectin expression was characterized by the increase in the number of tethered neutrophils under flow. Furthermore, DL-Hcy was

shown to increase the production of IL-8, which is known to be stored along with P-selectin in WPB. The fact that DL-Hcy induced increase in IL-8 concentration confirms that DL-Hcy induced the exocytosis of WPB, the storage vesicle of IL-8, to enable the release of this protein. WPB exocytosis will also lead to both release of P-selectin and its expression on EC surface. Therefore, the increase in IL-8 levels provides another indirect evidence that DL-Hcy induced the expression of P-selectin. Furthermore, DL-Hcy has been shown to decrease NO bioavailability (Stuhlinger *et al.*, 2001; Upchurch *et al.*, 1997), which is known to be a negative regulator of WPB exocytosis. This strengthens the conclusion that DL-Hcy might indirectly modulate WPB exocytosis by decreasing NO level. This conclusion provides another explanation for DL-Hcy-induced P-selectin up-regulation since WPB is the main storage for P-selectin and DL-Hcy enhances the exocytosis of this storage vesicle.

Collectively all of these observations could be considered as markers of EC activation/dysfunction. The up-regulation of these CAM and chemokine made the EC susceptible to be involved in different interactions with leukocytes, which were observed in the *in vitro* flow system used in this study. Hence, long term treatment with DL-Hcy was able to induce the earliest events in atherosclerosis process under flow conditions. This reflects to some extent, the *in vivo* situation in hyperhomocysteinemic patients and therefore, indicates a key role for this amino acid as a primary risk factor in atherosclerosis. Furthermore, none of the observed effects was reproducible with Cys indicating the observed EC atherogenic effects were not due to osmotic stress but specific to the DL-Hcy. Furthermore, Cys has a similar structure to DL-Hcy including the same free thiol group and

therefore, as Cys was not able to mimic DL-Hcy effects that indicates the effects of DL-Hcy are not non specific effects due to its free thiol group.

Different signalling pathways are involved in mediating CAM and cytokine up-regulation including MAP kinases and strong evidence provided in the literature suggests a key role for the JNK pathway in mediating cytokine-mediated E-selectin and ICAM-1 expression (Miho *et al.*, 2005; Min and Pober, 1997). Published studies have shown that acute Hcy treatment induced JNK pathway activation (Cai *et al.*, 2000; Dong *et al.*, 2005; Zhang *et al.*, 2001b) and the data of this thesis extended these findings and provided an evidence that chronic exposure to DL-Hcy induces sustained up-regulation of the JNK pathway and therefore sustained activation of its substrate c-Jun. Interestingly enough, only JNK1 of JNK isoforms was activated by chronic DL-Hcy, which is known to preferentially activate the substrate c-Jun compared to JNK2 (Sabapathy *et al.*, 2004) while TNF-α activated both JNK1 and JNK2. Furthermore, once this pathway was blocked, using a JNK specific inhibitor, DL-Hcy was not able to induce E-selectin or ICAM-1 expression and therefore interactions between neutrophils and EC under flow conditions were prevented. These findings are in line with others (Miho *et al.*, 2005; Min and Pober, 1997) who showed that E-selectin and ICAM-1 up-regulation in response to acute treatment with proinflammatory mediators happened through the JNK pathway. However, these CAM levels dropped back to the basal level after few hours of induction while, in this study E-selectin and ICAM-1 were still expressed after 5-9 days of DL-Hcy treatment indicating that DL-Hcy induced sustained expression of these CAM. This could be due to sustained activation of MKKK, the upstream kinase of the JNK pathway, or due to deactivation of MAPK phoasphatase, both of which induce a sustained activation of the JNK pathway.

Further work is still needed to reveal the mechanism of Hcy-induced sustained EC activation.

Furthermore, the inhibition of P-selectin expression (characterized by the number of tethered neutrophils under flow) in response to JNK inhibition was unexpected. Considering that P-selectin is presynthesized and stored in WPB, it could be concluded that JNK is involved either in *de novo* synthesis of P-selectin and/or in the WPB exocytosis, a process required for P-selectin surface expression. Collectively, this novel data shows that DL-Hcy induced up-regulation of different CAM by different mechanisms including the activation of the JNK pathway and altering vesicle trafficking within EC.

These findings suggest a potential therapeutic target for treating and/or protecting hyperhomocysteinemic patients from atherosclerosis by targeting the JNK pathway using specific inhibitors. However, the limitation would be that blocking such pathway would suppress different kinds of inflammatory responses including those which are vital for the survival of the whole organism such as those against invading organisms. Therefore, to avoid such side effects, these inhibitors would need to target specific cells at specific time points. The data illustrated in this study shows that DL-Hcy specifically activated JNK1; while TNF-α activated both JNK1 and JNK2. This might suggest that developing specific inhibitors against JNK1 might be a potential solution to avoid the side effects of JNK general inhibitors and therefore, avoiding blockage vital inflammatory responses.

It has been suggested that Hcy induces its atherogenic effects leading to EC activation/dysfunction via different mechanisms. Hcy was shown to induce the expression of several ER stress response genes (Kokame *et al.,* 1996) and also was suggested to induce

ER stress by disturbing disulphide bond formation and therefore leading to UPR as a cellular survival response (Kokame *et al.*, 1996; Outinen *et al.*, 1998; Outinen *et al.*, 1999). However, severe and prolonged ER stress elicited by Hcy and failed or prolonged UPR were suggested to have several atherogenic consequences including lipid dysregulation, programmed cell death and inflammation. Forming disulphide bonds between Hcy and Cys molecules was suggested also to disturb some enzymes which have free Cys groups like eNOS or DDAH and therefore disrupting their efficiency leading in both cases to decrease in the bioavailable NO (Boger, 2003; Boger *et al.*, 2001; Dayal and Lentz, 2005). Furthermore, it has been suggested that Hcy induces the production of ROS and/or disrupt antioxidant enzymes leading to cellular oxidative stress which was suggested to account for most intracellular Hcy effects (Au-Yeung *et al.*, 2004; Handy *et al.*, 2005). Chronic treatment with DL-Hcy in this study induced significant increases in ROS production in HUVEC leading to sustained oxidative stress. However, antioxidants like NAC were able to suppress ROS generation by DL-Hcy and also managed to reverse DL-Hcy-induced JNK activation. Therefore, once ROS generation was returned to basal levels, DL-Hcy was not able to activate the JNK pathway which indicates strongly that oxidative stress is the mechanism by which chronic DL-Hcy treatment activates the JNK pathway. Furthermore, treatment of the cells with DL-Hcy and the antioxidants NAC or vitamin C reversed DL-Hcy-induced EC:neutrophil interactions under flow conditions indicating that the CAM which mediated DL-Hcy effects under flow were not up-regulated in the absence of elevated ROS. These data could be summarized by suggesting that DL-Hcy induces continuous ROS generation which leads to sustained JNK pathway activation. Subsequently, both activated JNK and ROS might lead to up-regulation of different CAM

and chemokines which together mediate the EC:leukocyte interactions under flow. The fact that antioxidants inhibited DL-Hcy-induced JNK activation and inhibited DL-Hcy-induced interactions under flow, collectively suggest that ROS were responsible for activating the JNK pathway and therefore, inducing up-regulation of several CAM and potentially chemokines which mediated EC:neutrophil interactions under flow conditions.

These findings indicate the importance of antioxidants in reversing DL-Hcy atherogenic effects and therefore present a possible therapeutic approach to protect hHcy patients from CVD risk. Even though clinical trials using antioxidants have not so far been successful in decreasing the risk of CVD (Paolini *et al.*, 2003; Stanner *et al.*, 2004), our data agree with the findings of many *in vivo* and *in vitro* studies which indicate that restoring the redox balance help in improving EC functions and in reversing Hcy-induced EC activation/dysfunction (Chambers *et al.*, 1999; Nappo *et al.*, 1999). The reason for this contradiction is still unclear but could be that the use of only one antioxidant in some of these trials which might not be able to overcome the oxidative stress or could be due to the low dose used in *in vivo* studies compared to those used in *in vitro* studies. This issue is still in debate and needs more studies to uncover the efficiency of such treatment.

It has been shown so far that DL-Hcy induces ROS generation. However, the question which remains unanswered is how DL-Hcy induces ROS generation. Cys which shares the same free thiol group with Hcy was not able to induce ROS generation. This indicates that the Hcy-mediated ROS production is not due to the auto-oxidation of its thiol group but might be due to direct effects on ROS-producing enzymes or ROS-scavenging enzymes. Different theories have been suggested (Figure 8) including that Hcy decreases the expression of antioxidant enzymes such as glutathione peroxidase (Handy *et al.*, 2005),

Hcy decreases the bioavailability of NO or Hcy induces eNOS uncoupling and the production of superoxide anions at the expense of NO (Topal *et al.*, 2004). The results of this study along with the published data can be gathered in a possible theory including minor and major routes for ROS generation (Figure 52). Hcy activates NADPH oxidase which will start producing $O_2^{\bullet-}$. It was suggested this would be a minor route because that will oxidize BH_4 to BH_2 therefore, inhibiting NO-generating activity of eNOS and inducing its $O_2^{\bullet-}$-generating activity (Zhang *et al.*, 2000). Hcy was also shown to decrease the intracellular uptake of L-arginine in addition to activate arginase which is responsible for degrading L-arginine (Forstermann and Munzel, 2006) thus could reduce the intracellular levels of arginine (Jin *et al.*, 2007). Lack of BH_4 and arginine leads to eNOS uncoupling which will then start producing $O_2^{\bullet-}$ and that will be the major route. This $O_2^{\bullet-}$ interacts with NO to form peroxinitrite which is a very potent oxidant. $O_2^{\bullet-}$ produced by NADPH oxidase was suggested to work as an upstream for inducing more production of $O_2^{\bullet-}$ by other enzymatic systems (Gao and Mann, 2009). Therefore, NADPH oxidase could be the initial source of ROS and thereafter, uncoupled eNOS contributes to amplify the $O_2^{\bullet-}$ generation within Hcy-treated. Furthermore, Hcy ability to inhibit different antioxidant systems in the cell will amplify the effects of produced ROS.

In addition to generating superoxide anions, uncoupled eNOS has been shown to stop generating NO which is known to down-regulate the expression of CAM (Armstead *et al.*, 1997; Spiecker *et al.*, 1998; Zampolli *et al.*, 2000) and hence, inhibit leukocyte adhesion on EC. Therefore, the decrease in the bioavailable NO might play an important role in amplifying Hcy-induced EC activation which might worsen the Hcy-induced injury.

Thus, the potential therapeutic effects of NO in reversing Hcy effects and restoring EC quiescence were investigated.

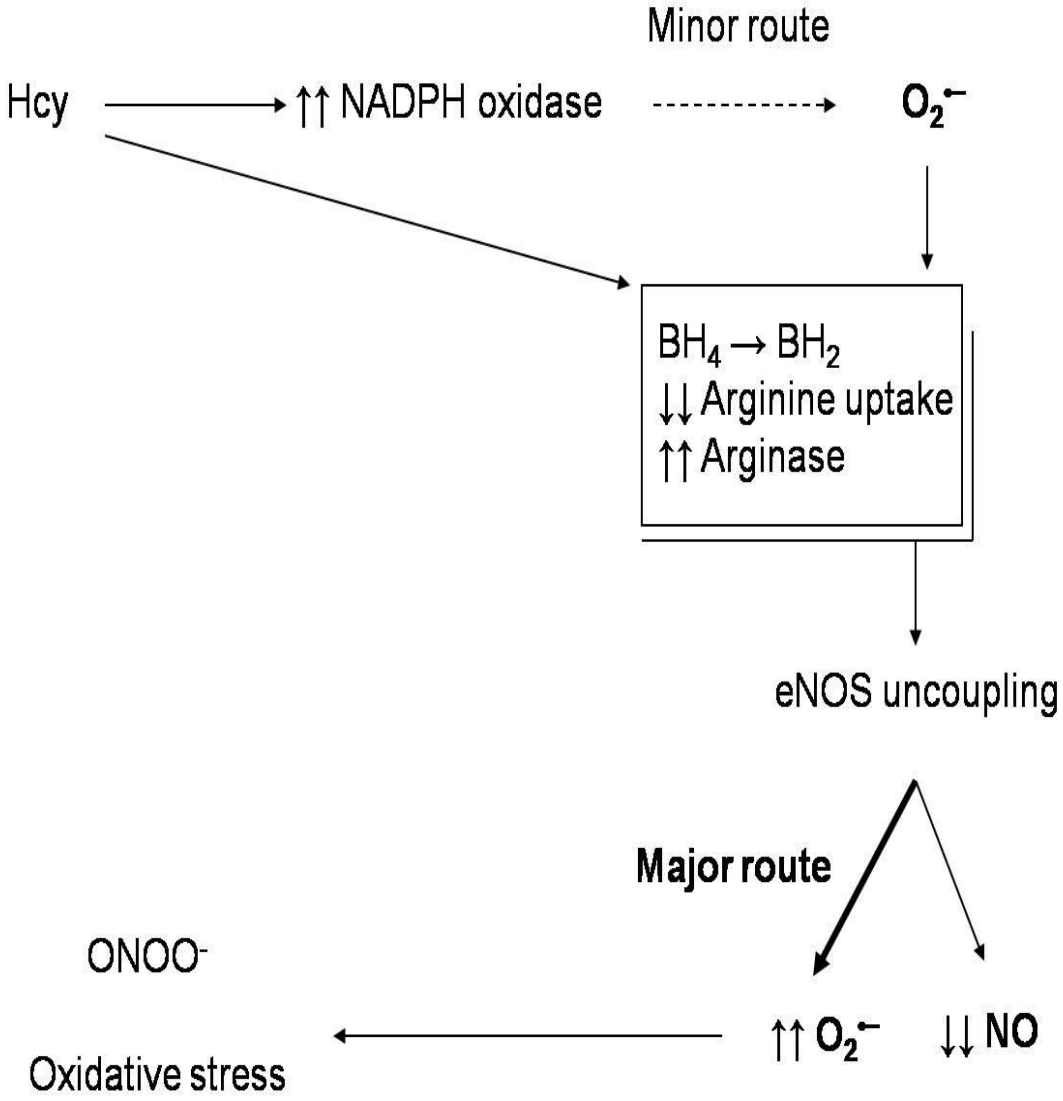


Figure 52: Minor and major routes for ROS production induced by Hcy.

Most previous studies have shown a role of NO in regulating *de novo* synthesis of CAM. Surprisingly, short incubations with exogenous NO conducted in this thesis were able to reverse the effects of chronic DL-Hcy treatment on EC under flow and no EC:neutrophil interactions were recorded. NO requires a number of hours to alter the *de novo* synthesis of CAM like E-selectin and ICAM-1 and thus to down-regulate their expression. Therefore, the short-term incubation with NO in this study (10min) was unlikely to be enough to down-regulate the expression of these two CAM and hence, they are likely to be still expressed on the EC surface. However, the question remains as to why neutrophils did not interact with DL-Hcy/NO-treated cells. NO can nitrosylate some proteins altering their activity and included in those proteins are the NSF superfamily which mediate WPB exocytosis. Therefore, within 10min of NO treatment, we believe these proteins were nitrosylated leading to inhibition of WPB exocytosis. Both P-selectin and IL-8 are stored in WPB and require vesicle exocytosis to be released. Collectively, short-time treatment with NO might have inhibited WPB exocytosis and thus inhibited P-selectin surface expression, the main mediator of tethering, and IL-8 release, one of the main chemokines required for leukocyte recruitment. Therefore, NO short treatment prevented neutrophils tethering to EC and this step was showed to be essential for leukocyte activation and their integrin conformational change to the adhesive form (Lawrence and Springer, 1991; Lorant *et al.*, 1993), both of which are required for the rolling and firm adhesion stages to happen. Therefore, none of the neutrophils were able to roll directly or adhere without being activated through the tethering step first. Therefore, all the stages were inhibited and neutrophils did not interact at all with EC. It could be concluded that short-term treatment with NO inhibited DL-Hcy atherogenic effects and

these findings suggest possible use of exogenous NO in treating or protecting hyperhomocysteinemic patients from atherosclerosis risk.

The last part of this study was to investigate the involvement of chemokines and cytokines in mediating DL-Hcy effects. Chronic DL-Hcy induced a significant increase in the expression of IL-8 protein but did not up-regulate mRNA. That means DL-Hcy induced the translation of IL-8 mRNA to the protein, which is an essential chemokine that helps in recruiting leukocytes to activated EC. IL-8 expression in addition to CAM up-regulation work together on mediating DL-Hcy-induced HUVEC:neutrophil interactions under flow conditions. Furthermore, chronic treatment with DL-Hcy induced increased expression of TNF-α mRNA but not its protein. TNF-α is a very well characterized proinflammatory cytokine and it has been shown to induce ROS generation (Gu *et al.*, 2002) which is shown to inhibit JNK phosphatases (Chen *et al.*, 2001a) leading to JNK activation and thereafter up-regulation of different CAM and chemokines. TNF-α might have peaked in the early few hours after DL-Hcy treatment and then dropped down to an undetectable level. This early increase could have induced the expression of several proinflammatory cytokines which mediated and/or amplified DL-Hcy atherogenic effects. The findings of this study are in line with most published studies which failed to record any TNF-α in EC (Dalal *et al.*, 2003; Kerkeni *et al.*, 2006; Nikitina *et al.*, 1997). It might be more helpful to study the expression of other proinflammatory cytokines like IL-1 which is already known to be expressed in EC.

Even though, different clinical trials (explained earlier in section 1.1.5.4.2) have used vitamin B6, B12 or folate to lower Hcy levels, they failed to report any benefit of this reduction on CVD risk. However, the results of this thesis contradict with the finding of

these clinical trials and they illustrate a clear role for Hcy in inducing EC damage and initiating for atherosclerosis. Furthermore, three possible therapeutic approaches were discussed and suggested from results obtained in this thesis, the use of JNK inhibitors which would be not easy to control as there is a need to target specific cells at specific times in order not to disturb the necessary inflammatory response. However, targeting specific JNK isoforms, such as JNK1 might present a possible solution to avoid the unwanted side effects. The second approach is by using NO which has potentially a great application in patients with MI as a quick intervention. However, the long term use of exogenous NO donors in a protective manner has not been fully investigated yet. Antioxidant treatment presents the third approach which is safe and easy to use; however, the clinical trials were not as successful as the *in vitro* studies. More studies are still needed to evaluate any combination or required doses for antioxidants. The negative outcome of clinical trials using antioxidants in CVD patients (explained in section 1.3.4) might suggest an additional therapeutic option by targeting specific enzymes like NADPH oxidase or eNOS which could provide more efficient solution to restore antioxidant system to protect against or reverse some effects of CVD.

To summarize, the results of this thesis provide, for the first time, clear evidence that chronic treatment with DL-Hcy induced EC activation which was characterized by sustained activation of the JNK pathway through an ROS-mediated mechanism. Furthermore, activation of the JNK pathway led to continuous up-regulation of different CAM and chemokines secretion. Thereafter, surface expressed CAM and secreted chemokines mediated different sorts of interactions between EC and neutrophils under flow representing the earliest interaction in atherosclerosis progress. The long-term treatment

with DL-Hcy along with the *in vitro* flow model used in this thesis together provided a much reliable results than previous experimental approaches which used short-term treatment with DL-Hcy and/or evaluated DL-Hcy effects under static conditions. The outcome of this study clearly illustrates the importance of DL-Hcy as a primary risk factor in atherosclerosis. Different studies have suggested different approaches to lowering Hcy levels in patients at high risk by using vitamins such as vitamin B12 and folate (Bonaa *et al.*, 2006; Lonn *et al.*, 2006). However, these studies were of no benefit in reducing cardiovascular risk, this may be attributed to the complex metabolic networks involving homocysteine and vitamins (Loscalzo, 2006). This study evaluated the efficiency of different therapeutic approaches to reverse Hcy-induced activation of EC under static and flow conditions, such as using antioxidants, NO donors or being more specific by targeting some JNK isoforms. Despite showing Hcy as a primary risk factor that is implicated in different atherogenic consequences, this study highlighted the possibility to protect or cure from Hcy effects.

7.2. Future studies

This thesis provides strong evidence for atherogenic effects of chronic Hcy. However and due to time and cost efficiency, there are some limitations to the work conducted in this thesis. Additional techniques could be used to answer more questions on the mechanisms of Hcy-induced EC activation and atherosclerotic initiation.

Firstly, using small interfering RNA (siRNA) technology to inhibit different gene expression provides a specific technique to selectively inhibit some proteins. siRNA could be used to inhibit the expression of each CAM individually and then study EC:leukocyte interaction under flow conditions. That would provide clear evidence for the role of each CAM in each stage of EC:leukocyte interaction and explore any overlapping between them in mediating the different stages. Furthermore, since it was shown that EC are unlikely to produce TNF-α, the involvement of other proinflammatory cytokines, known to be expressed in EC, such as IL-1 could be a good aspect of study using the same siRNA technology. That would provide a clear evidence of the actual role of proinflammatory cytokines in Hcy-induced atherogenic effects. Furthermore, using the same technique to inhibit JNK might give a more reliable answer for its role in CAM up-regulation than using selective inhibitors as some researchers argue about the specificity of these inhibitors.

Secondly, the dramatic and instant effects of NO on Hcy-induced EC:neutrophil interactions need more investigation. The surface expression of different CAM after a short-term NO treatment could be further investigated to identify NO ability to induce the shedding of these CAM. Immunofluorescence techniques could be used to study these CAM expression using direct immunostaining that was used in this thesis or using more sensitive techniques such as fluorescence-activated cell sorting (FACS). Furthermore, WPB

exocytosis could be studied at real time by transfecting EC with the green fluorescent protein (GFP) P-selectin probe which will identify P-selectin and therefore allow visualizing vesicle trafficking in real time. That would be very helpful in studying the inhibitory effects of NO on this process. Nitrosylation could be further investigated by using reducing agents with less damaging effects than DTT, to study whether these compounds can reverse NO inhibitory effects. That could give a supporting evidence for the nitrosylation effects. Furthermore, protein nitrosylation after exposure to exogenous NO donors could be detected using the biotin switch assay and this technique could be made more specific by immunoprecipitating JNK first and then using the biotin switch assay to detect any nitrosylation of JNK. Finally, Better flow model can be used like that developed by Sheikh *et al* (2003) where cells were cultured under flow. That model gives the opportunity to study the effects of chronic Hcy on shear stress induced NO production and whether shear stress anti-atherogenic effects might overcome Hcy atherogenic consequences.

REFERENCES

Abbassi, O., Kishimoto, T. K., McIntire, L. V., Anderson, D. C., and Smith, C. W. (1993). E-selectin supports neutrophil rolling in vitro under conditions of flow. J Clin Invest *92*, 2719-2730.

Abdel Aziz, M. T., Fouad, H. H., Mohsen, G. A., Mansour, M., and Abdel Ghaffar, S. (2001). TNF-alpha and homocysteine levels in type 1 diabetes mellitus. East Mediterr Health J *7*, 679-688.

Adams, M. R., Jessup, W., Hailstones, D., and Celermajer, D. S. (1997). L-arginine reduces human monocyte adhesion to vascular endothelium and endothelial expression of cell adhesion molecules. Circulation *95*, 662-668.

Ahluwalia, A., Foster, P., Scotland, R. S., McLean, P. G., Mathur, A., Perretti, M., Moncada, S., and Hobbs, A. J. (2004). Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of P-selectin expression and leukocyte recruitment. Proc Natl Acad Sci U S A *101*, 1386-1391.

Ahmad, M., Theofanidis, P., and Medford, R. M. (1998). Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-alpha. J Biol Chem *273*, 4616-4621.

Akalin, A., Alatas, O., and Colak, O. (2008). Relation of plasma homocysteine levels to atherosclerotic vascular disease and inflammation markers in type 2 diabetic patients. Eur J Endocrinol *158*, 47-52.

Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. Biochem J *357*, 593-615.

Alkhoury, K., Parkin, S., and Graham, A. (2009a). Abstracts: JNK MAP Kinase pathway is important in neutrophil:endothelial cell interactions in response to chronic homocysteine treatment Heart *95*, e1-.

Alkhoury, K., Parkin, S. M., and Graham, A. M. (2009b). Oxidative stress mediates homocysteine proatherosclerotic effects on human endothelial cells through a MAP kinase pathway. FASEB J *23*, 762.

Alvarez-Maqueda, M., El Bekay, R., Monteseirin, J., Alba, G., Chacon, P., Vega, A., Santa Maria, C., Tejedo, J. R., Martin-Nieto, J., Bedoya, F. J., *et al.* (2004). Homocysteine enhances superoxide anion release and NADPH oxidase assembly by human neutrophils. Effects on MAPK activation and neutrophil migration. Atherosclerosis *172*, 229-238.

Amezcua, J. L., Palmer, R. M., de Souza, B. M., and Moncada, S. (1989). Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. Br J Pharmacol *97*, 1119-1124.

Apostolopoulos, J., Davenport, P., and Tipping, P. G. (1996). Interleukin-8 Production by Macrophages From Atheromatous Plaques. Arterioscler Thromb Vasc Biol *16*, 1007-1012.

Armstead, V. E., Minchenko, A. G., Schuhl, R. A., Hayward, R., Nossuli, T. O., and Lefer, A. M. (1997). Regulation of P-selectin expression in human endothelial cells by nitric oxide. Am J Physiol *273*, H740-746.

Arribas, M., and Cutler, D. F. (2000). Weibel-Palade body membrane proteins exhibit differential trafficking after exocytosis in endothelial cells. Traffic *1*, 783-793.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Atmaca, G. (2004). Antioxidant effects of sulfur-containing amino acids. Yonsei Med J *45*, 776-788.

Au-Yeung, K. K., Woo, C. W., Sung, F. L., Yip, J. C., Siow, Y. L., and O, K. (2004). Hyperhomocysteinemia activates nuclear factor-kappaB in endothelial cells via oxidative stress. Circ Res *94*, 28-36.

Austin, R. C., Lentz, S. R., and Werstuck, G. H. (2004). Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. Cell Death Differ *11 Suppl 1*, S56-64.

Baehner, R. L., Boxer, L. A., and Davis, J. (1976). The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. Blood *48*, 309-313.

Bai, Y. P., Liu, Y. H., Chen, J., Song, T., You, Y., Tang, Z. Y., Li, Y. J., and Zhang, G. G. (2007). Rosiglitazone attenuates NF-kappaB-dependent ICAM-1 and TNF-alpha production caused by homocysteine via inhibiting ERK1/2/p38MAPK activation. Biochem Biophys Res Commun *360*, 20-26.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem J *371*, 199-204.

Ballinger, S. W., Patterson, C., Knight-Lozano, C. A., Burow, D. L., Conklin, C. A., Hu, Z., Reuf, J., Horaist, C., Lebovitz, R., Hunter, G. C., *et al.* (2002). Mitochondrial integrity and function in atherogenesis. Circulation *106*, 544-549.

Bargatze, R. F., Kurk, S., Butcher, E. C., and Jutila, M. A. (1994). Neutrophils roll on adherent neutrophils bound to cytokine-induced endothelial cells via L-selectin on the rolling cells. J Exp Med *180*, 1785-1792.

Barr, R. K., and Bogoyevitch, M. A. (2001). The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). Int J Biochem Cell Biol *33*, 1047-1063.

Bates, C. J., Mansoor, M. A., van der Pols, J., Prentice, A., Cole, T. J., and Finch, S. (1997). Plasma total homocysteine in a representative sample of 972 British men and women aged 65 and over. Eur J Clin Nutr *51*, 691-697.

Baud, V., and Karin, M. (2001). Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol *11*, 372-377.

Bellamy, M. F., McDowell, I. F., Ramsey, M. W., Brownlee, M., Bones, C., Newcombe, R. G., and Lewis, M. J. (1998). Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. Circulation *98*, 1848-1852.

Bennet, A. M., van Maarle, M. C., Hallqvist, J., Morgenstern, R., Frostegard, J., Wiman, B., Prince, J. A., and de Faire, U. (2006). Association of TNF-alpha serum levels and

TNFA promoter polymorphisms with risk of myocardial infarction. Atherosclerosis *187*, 408-414.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., *et al.* (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A *98*, 13681-13686.

Berk, B. C. (2008). Atheroprotective signaling mechanisms activated by steady laminar flow in endothelial cells. Circulation *117*, 1082-1089.

Bevilacqua, M. P. (1993). Endothelial-leukocyte adhesion molecules. Annu Rev Immunol *11*, 767-804.

Bevilacqua, M. P., and Nelson, R. M. (1993). Selectins. J Clin Invest *91*, 379-387.

Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr., and Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science *243*, 1160-1165.

Blann, A. D., Nadar, S. K., and Lip, G. Y. (2003). The adhesion molecule P-selectin and cardiovascular disease. Eur Heart J *24*, 2166-2179.

Boers, G. H., Smals, A. G., Trijbels, F. J., Fowler, B., Bakkeren, J. A., Schoonderwaldt, H. C., Kleijer, W. J., and Kloppenborg, P. W. (1985). Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med *313*, 709-715.

Boger, R. H. (2003). The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. Cardiovasc Res *59*, 824-833.

Boger, R. H., Lentz, S. R., Bode-Boger, S. M., Knapp, H. R., and Haynes, W. G. (2001). Elevation of asymmetrical dimethylarginine may mediate endothelial dysfunction during experimental hyperhomocyst(e)inaemia in humans. Clin Sci (Lond) *100*, 161-167.

Bogoyevitch, M. A. (2005). Therapeutic promise of JNK ATP-noncompetitive inhibitors. Trends Mol Med *11*, 232-239.

Bolander-Gouaille, C. (2002). Focus on homocysteine and the vitamins involved in its metabolism, 2nd enlarged and rev. edn (Paris ; London: Springer).

Bonaa, K. H., Njolstad, I., Ueland, P. M., Schirmer, H., Tverdal, A., Steigen, T., Wang, H., Nordrehaug, J. E., Arnesen, E., and Rasmussen, K. (2006). Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med *354*, 1578-1588.

Bonetti, P. O., Lerman, L. O., and Lerman, A. (2003). Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol *23*, 168-175.

Born, G. V. R., Rabelink, T., and Smith, T. (1998). Clinician's manual on endothelium and cardiovasculer disease (London: Science Press).

Bostom, A. G., and Lathrop, L. (1997). Hyperhomocysteinemia in end-stage renal disease: prevalence, etiology, and potential relationship to arteriosclerotic outcomes. Kidney Int *52*, 10-20.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yancopoulos, G. D. (1991). ERKs:

a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell *65*, 663-675.

Braunersreuther, V., Mach, F., and Steffens, S. (2007). The specific role of chemokines in atherosclerosis. Thromb Haemost *97*, 714-721.

Brevetti, G., Schiano, V., and Chiariello, M. (2008). Endothelial dysfunction: a key to the pathophysiology and natural history of peripheral arterial disease? Atherosclerosis *197*, 1-11.

Bruckdorfer, R. (2005). The basics about nitric oxide. Mol Aspects Med *26*, 3-31.

Budy, B., O'Neill, R., DiBello, P. M., Sengupta, S., and Jacobsen, D. W. (2006). Homocysteine transport by human aortic endothelial cells: identification and properties of import systems. Arch Biochem Biophys *446*, 119-130.

Butler, A. R., and Nicholson, R. (2003). Life, death and nitric oxide (Cambridge: Royal Society of Chemistry).

Cai, H., and Harrison, D. G. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res *87*, 840-844.

Cai, Y., Zhang, C., Nawa, T., Aso, T., Tanaka, M., Oshiro, S., Ichijo, H., and Kitajima, S. (2000). Homocysteine-responsive ATF3 gene expression in human vascular endothelial cells: activation of c-Jun NH(2)-terminal kinase and promoter response element. Blood *96*, 2140-2148.

Carlos, T. M., and Harlan, J. M. (1994). Leukocyte-endothelial adhesion molecules. Blood *84*, 2068-2101.

Carmel, R., and Jacobsen, D. W. (2001). Homocysteine in health and disease (Cambridge: Cambridge University Press).

Cavigelli, M., Li, W. W., Lin, A., Su, B., Yoshioka, K., and Karin, M. (1996). The tumor promoter arsenite stimulates AP-1 activity by inhibiting a JNK phosphatase. Embo J *15*, 6269-6279.

Celermajer, D. S. (1997). Endothelial dysfunction: does it matter? Is it reversible? J Am Coll Cardiol *30*, 325-333.

Chacko, G., Ling, Q., and Hajjar, K. A. (1998). Induction of acute translational response genes by homocysteine. Elongation factors-1alpha, -beta, and -delta. J Biol Chem *273*, 19840-19846.

Chambers, J. C., McGregor, A., Jean-Marie, J., and Kooner, J. S. (1998). Acute hyperhomocysteinaemia and endothelial dysfunction. Lancet *351*, 36-37.

Chambers, J. C., McGregor, A., Jean-Marie, J., Obeid, O. A., and Kooner, J. S. (1999). Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy. Circulation *99*, 1156-1160.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37-40.

Chang, P. Y., Lu, S. C., Lee, C. M., Chen, Y. J., Dugan, T. A., Huang, W. H., Chang, S. F., Liao, W. S., Chen, C. H., and Lee, Y. T. (2008). Homocysteine inhibits arterial endothelial cell growth through transcriptional downregulation of fibroblast growth factor-2 involving G protein and DNA methylation. Circ Res *102*, 933-941.

Channon, K. (2004). Tetrahydrobiopterin: Regulator of Endothelial Nitric Oxide Synthase in Vascular Disease. Trends in Cardiovascular Medicine *14*, 323-327.

Chen, P., Poddar, R., Tipa, E. V., Dibello, P. M., Moravec, C. D., Robinson, K., Green, R., Kruger, W. D., Garrow, T. A., and Jacobsen, D. W. (1999). Homocysteine metabolism in cardiovascular cells and tissues: implications for hyperhomocysteinemia and cardiovascular disease. Adv Enzyme Regul *39*, 93-109.

Chen, Y. R., Shrivastava, A., and Tan, T. H. (2001a). Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate. Oncogene *20*, 367-374.

Chen, Z., Karaplis, A. C., Ackerman, S. L., Pogribny, I. P., Melnyk, S., Lussier-Cacan, S., Chen, M. F., Pai, A., John, S. W., Smith, R. S., *et al.* (2001b). Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. Hum Mol Genet *10*, 433-443.

Chin, J. H., Azhar, S., and Hoffman, B. B. (1992). Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. J Clin Invest *89*, 10-18.

Chiu, J. J., Chen, L. J., Lee, C. I., Lee, P. L., Lee, D. Y., Tsai, M. C., Lin, C. W., Usami, S., and Chien, S. (2007). Mechanisms of induction of endothelial cell E-selectin expression by smooth muscle cells and its inhibition by shear stress. Blood *110*, 519-528.

Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., McEver, R. P., Pober, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S., *et al.* (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood *91*, 3527-3561.

Conran, N., Gambero, A., Ferreira, H. H., Antunes, E., and de Nucci, G. (2003). Nitric oxide has a role in regulating VLA-4-integrin expression on the human neutrophil cell surface. Biochem Pharmacol *66*, 43-50.

Cooper, D. (2000) Stess activated signalling pathways in human endothelial cells : an investigation of NF-kB, c-Jun N terminal kinase and p38 MAP kinase signalling pathways in human saphenous vein endothelial cells exposed to hypoxia and cytokines, Ph.D. thesis. Typescript., Bradford,.

Coxon, A., Tang, T., and Mayadas, T. N. (1999). Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo. A role for granulocyte/macrophage colony-stimulating factor. J Exp Med *190*, 923-934.

Crowe, D. L., Tsang, K. J., and Shemirani, B. (2001). Jun N-terminal kinase 1 mediates transcriptional induction of matrix metalloproteinase 9 expression. Neoplasia *3*, 27-32.

D'Angelo, A., and Selhub, J. (1997). Homocysteine and thrombotic disease. Blood *90*, 1-11.

Dalal, S., Parkin, S. M., Homer-Vanniasinkam, S., and Nicolaou, A. (2003). Effect of homocysteine on cytokine production by human endothelial cells and monocytes. Ann Clin Biochem *40*, 534-541.

Dardik, R., Savion, N., Gal, N., and Varon, D. (2002). Flow conditions modulate homocysteine induced changes in the expression of endothelial cell genes associated with cell-cell interaction and cytoskeletal rearrangement. Thromb Haemost *88*, 1047-1053.

Davidson, S. M., and Duchen, M. R. (2007). Endothelial mitochondria: contributing to vascular function and disease. Circ Res *100*, 1128-1141.

Davignon, J., and Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. Circulation *109*, III27-32.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

Dayal, S., Arning, E., Bottiglieri, T., Boger, R. H., Sigmund, C. D., Faraci, F. M., and Lentz, S. R. (2004). Cerebral vascular dysfunction mediated by superoxide in hyperhomocysteinemic mice. Stroke *35*, 1957-1962.

Dayal, S., Brown, K. L., Weydert, C. J., Oberley, L. W., Arning, E., Bottiglieri, T., Faraci, F. M., and Lentz, S. R. (2002). Deficiency of glutathione peroxidase-1 sensitizes hyperhomocysteinemic mice to endothelial dysfunction. Arterioscler Thromb Vasc Biol *22*, 1996-2002.

Dayal, S., and Lentz, S. R. (2005). ADMA and hyperhomocysteinemia. Vasc Med *10 Suppl 1*, S27-33.

De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., Jr., Shin, W. S., and Liao, J. K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest *96*, 60-68.

Deanfield, J. E., Halcox, J. P., and Rabelink, T. J. (2007). Endothelial function and dysfunction: testing and clinical relevance. Circulation *115*, 1285-1295.

Dejana, E., Corada, M., and Lampugnani, M. G. (1995). Endothelial cell-to-cell junctions. Faseb J *9*, 910-918.

den Heijer, M., Koster, T., Blom, H. J., Bos, G. M., Briet, E., Reitsma, P. H., Vandenbroucke, J. P., and Rosendaal, F. R. (1996). Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. N Engl J Med *334*, 759-762.

Derakhshan, B., Hao, G., and Gross, S. S. (2007). Balancing reactivity against selectivity: the evolution of protein S-nitrosylation as an effector of cell signaling by nitric oxide. Cardiovasc Res *75*, 210-219.

Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025-1037.

Desai, A., Lankford, H. A., and Warren, J. S. (2001). Homocysteine augments cytokine-induced chemokine expression in human vascular smooth muscle cells: implications for atherogenesis. Inflammation *25*, 179-186.

Dimmeler, S., Haendeler, J., Galle, J., and Zeiher, A. M. (1997). Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. Circulation *95*, 1760-1763.

Dong, F., Zhang, X., Li, S. Y., Zhang, Z., Ren, Q., Culver, B., and Ren, J. (2005). Possible involvement of NADPH oxidase and JNK in homocysteine-induced oxidative stress and apoptosis in human umbilical vein endothelial cells. Cardiovasc Toxicol *5*, 9-20.

Droge, W. (2002). Free radicals in the physiological control of cell function. Physiol Rev *82*, 47-95.

Dudman, N. P., and Hale, S. E. (1997). Endothelial- and leukocyte-mediated mechanisms in homocysteine-associated occlusive vascular disease, In Homocysteine Metabolism: From Basic Science to Clinical Medicine, R. H. Graham I, Rosenberg IH, Ueland PM, ed. (Boston: Kluwer Academic), pp. 267–271.

Dudman, N. P., Temple, S. E., Guo, X. W., Fu, W., and Perry, M. A. (1999). Homocysteine enhances neutrophil-endothelial interactions in both cultured human cells and rats In vivo. Circ Res *84*, 409-416.

Dunne, J. L., Ballantyne, C. M., Beaudet, A. L., and Ley, K. (2002). Control of leukocyte rolling velocity in TNF-alpha-induced inflammation by LFA-1 and Mac-1. Blood *99*, 336-341.

Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A., and Springer, T. A. (1986). Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J Immunol *137*, 245-254.

Eccles, K. A. (2006) Investigation of endothelial cell: leukocyte interactions in acute and chronic inflammation : an investigation of the effects of conditions of hypoxia/reoxygenation, chronic exposure to pathological concentrations of homocysteine and pre-treatment of endothelial cells with simvastatin ... conditions of physiological flow, Ph.D. thesis. Typescript., Bradford.

Eccles, K. A., Sowden, H., Porter, K. E., Parkin, S. M., Homer-Vanniasinkam, S., and Graham, A. M. (2008). Simvastatin alters human endothelial cell adhesion molecule expression and inhibits leukocyte adhesion under flow. Atherosclerosis.

Edirimanne, V. E., Woo, C. W., Siow, Y. L., Pierce, G. N., Xie, J. Y., and O, K. (2007). Homocysteine stimulates NADPH oxidase-mediated superoxide production leading to endothelial dysfunction in rats. Can J Physiol Pharmacol *85*, 1236-1247.

Elangbam, C. S., Qualls, C. W., Jr., and Dahlgren, R. R. (1997). Cell adhesion molecules--update. Vet Pathol *34*, 61-73.

Eligini, S., Barbieri, S. S., Cavalca, V., Camera, M., Brambilla, M., De Franceschi, M., Tremoli, E., and Colli, S. (2005). Diversity and similarity in signaling events leading to

rapid Cox-2 induction by tumor necrosis factor-alpha and phorbol ester in human endothelial cells. Cardiovasc Res *65*, 683-693.

Ennis, B. W., Fultz, K. E., Smith, K. A., Westwick, J. K., Zhu, D., Boluro-Ajayi, M., Bilter, G. K., and Stein, B. (2005). Inhibition of tumor growth, angiogenesis, and tumor cell proliferation by a small molecule inhibitor of c-Jun N-terminal kinase. J Pharmacol Exp Ther *313*, 325-332.

Esper, R. J., Nordaby, R. A., Vilarino, J. O., Paragano, A., Cacharron, J. L., and Machado, R. A. (2006). Endothelial dysfunction: a comprehensive appraisal. Cardiovasc Diabetol *5*, 4.

Ewadh, M. J., Tudball, N., and Rose, F. A. (1990). Homocysteine uptake by human umbilical vein endothelial cells in culture. Biochim Biophys Acta *1054*, 263-266.

Farooq, A., and Zhou, M. M. (2004). Structure and regulation of MAPK phosphatases. Cell Signal *16*, 769-779.

Feghali, C. A., and Wright, T. M. (1997). Cytokines in acute and chronic inflammation. Front Biosci *2*, d12-26.

Fei, J., Viedt, C., Soto, U., Elsing, C., Jahn, L., and Kreuzer, J. (2000). Endothelin-1 and smooth muscle cells: induction of jun amino-terminal kinase through an oxygen radical-sensitive mechanism. Arterioscler Thromb Vasc Biol *20*, 1244-1249.

Ferrell, J. E., Jr. (1996). Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem Sci *21*, 460-466.

Ferretti, G., Bacchetti, T., Moroni, C., Vignini, A., Nanetti, L., and Curatola, G. (2004). Effect of homocysteinylation of low density lipoproteins on lipid peroxidation of human endothelial cells. J Cell Biochem *92*, 351-360.

Finkelstein, J. D. (1998). The metabolism of homocysteine: pathways and regulation. Eur J Pediatr *157 Suppl 2*, S40-44.

Fleming, I., Bauersachs, J., and Busse, R. (1997). Calcium-dependent and calcium-independent activation of the endothelial NO synthase. J Vasc Res *34*, 165-174.

Forstermann, U. (2008). Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. Nat Clin Pract Cardiovasc Med *5*, 338-349.

Forstermann, U., and Munzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation *113*, 1708-1714.

Frank, J., Beck, S. C., Flaccus, A., and Biesalski, H. K. (2007). No evidence for prooxidative effects of homocysteine in vascular endothelial cells. Eur J Nutr *46*, 286-292.

Frei, B. (1999). On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. Proc Soc Exp Biol Med *222*, 196-204.

Furchgott, R. F. (1998). Nitric oxide: from basic research on isolated blood vessels to clinical relevance in diabetes. An R Acad Nac Med (Madr) *115*, 317-331.

Furie, M. B., Tancinco, M. C., and Smith, C. W. (1991). Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1

inhibit chemoattractant-stimulated neutrophil transendothelial migration in vitro. Blood *78*, 2089-2097.

Fuster, V., Fayad, Z. A., and Badimon, J. J. (1999). Acute coronary syndromes: biology. The Lancet *353*, s5-s9.

Gahmberg, C. G., Valmu, L., Tian, L., Kotovuori, P., Fagerholm, S., Kotovuori, A., Kantor, C., and Hilden, T. (1999). Leukocyte adhesion - a fundamental process in leukocyte physiology. Brazilian Journal of Medical and Biological Research *32*, 511-517.

Galkina, E., and Ley, K. (2009). Immune and inflammatory mechanisms of atherosclerosis (*). Annu Rev Immunol *27*, 165-197.

Gao, L., and Mann, G. E. (2009). Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling. Cardiovasc Res *82*, 9-20.

Gaston, B. M., Carver, J., Doctor, A., and Palmer, L. A. (2003). S-nitrosylation signaling in cell biology. Mol Interv *3*, 253-263.

Gauthier, T. W., Davenpeck, K. L., and Lefer, A. M. (1994). Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion. Am J Physiol *267*, G562-568.

Geisel, J., Jodden, V., Obeid, R., Knapp, J. P., Bodis, M., and Herrmann, W. (2003). Stimulatory effect of homocysteine on interleukin-8 expression in human endothelial cells. Clin Chem Lab Med *41*, 1045-1048.

Gerritsen, T., and Waisman, H. A. (1964). Homocystinuria, an Error in the Metabolism of Methionine. Pediatrics *33*, 413-420.

Gerszten, R. E., Garcia-Zepeda, E. A., Lim, Y.-C., Yoshida, M., Ding, H. A., Gimbrone, M. A., Luster, A. D., Luscinskas, F. W., and Rosenzweig, A. (1999). MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature *398*, 718-723.

Gey, K. F., Puska, P., Jordan, P., and Moser, U. K. (1991). Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. Am J Clin Nutr *53*, 326S-334S.

Goebeler, M., Kilian, K., Gillitzer, R., Kunz, M., Yoshimura, T., Brocker, E. B., Rapp, U. R., and Ludwig, S. (1999). The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. Blood *93*, 857-865.

Graham, A. M., Alkhoury, K., Naseem, K., and Parkin, S. M. (2009). Nitric oxide inhibition of chronic Hcy-induced endothelial cell (EC) activation is cGMP-independent. FASEB J *23*, 568.

Gryglewski, R., and Minuz, P. (2001). Nitric oxide : basic research and clinical applications (Amsterdam; Washington, D.C.: IOS Press).

Gu, Y., Xu, Y. C., Wu, R. F., Souza, R. F., Nwariaku, F. E., and Terada, L. S. (2002). TNFalpha activates c-Jun amino terminal kinase through p47(phox). Exp Cell Res *272*, 62-74.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996). Selective interaction of JNK protein kinase isoforms with transcription factors. Embo J *15*, 2760-2770.

Hahne, M., Jager, U., Isenmann, S., Hallmann, R., and Vestweber, D. (1993). Five tumor necrosis factor-inducible cell adhesion mechanisms on the surface of mouse endothelioma cells mediate the binding of leukocytes. J Cell Biol *121*, 655-664.

Haier, J., and Nicolson, G. L. (2000). Cell biology and clinical implications of adhesion molecules in colorectal diseases: colorectal cancers, infections and inflammatory bowel diseases. Clin Exp Metastasis *18*, 623-638.

Hajjar, K. A. (1993). Homocysteine-induced modulation of tissue plasminogen activator binding to its endothelial cell membrane receptor. J Clin Invest *91*, 2873-2879.

Hajjar, K. A. (2001). Homocysteine: a sulph'rous fire. J Clin Invest *107*, 663-664.

Hajjar, K. A., Mauri, L., Jacovina, A. T., Zhong, F., Mirza, U. A., Padovan, J. C., and Chait, B. T. (1998). Tissue plasminogen activator binding to the annexin II tail domain. Direct modulation by homocysteine. J Biol Chem *273*, 9987-9993.

Hall, J. P., Merithew, E., and Davis, R. J. (2000). c-Jun N-terminal kinase (JNK) repression during the inflammatory response? Just say NO. Proc Natl Acad Sci U S A *97*, 14022-14024.

Halliwell, B., and Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol *142*, 231-255.

Handy, D. E., Zhang, Y., and Loscalzo, J. (2005). Homocysteine down-regulates cellular glutathione peroxidase (GPx1) by decreasing translation. J Biol Chem *280*, 15518-15525.

Harada, A., Mukaida, N., and Matsushima, K. (1996). Interleukin 8 as a novel target for intervention therapy in acute inflammatory diseases. Mol Med Today *2*, 482-489.

Harpel, P. C., Chang, V. T., and Borth, W. (1992). Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. Proc Natl Acad Sci U S A *89*, 10193-10197.

Hartwell, D. W., Mayadas, T. N., Berger, G., Frenette, P. S., Rayburn, H., Hynes, R. O., and Wagner, D. D. (1998). Role of P-selectin cytoplasmic domain in granular targeting in vivo and in early inflammatory responses. J Cell Biol *143*, 1129-1141.

Hattori, R., Hamilton, K. K., Fugate, R. D., McEver, R. P., and Sims, P. J. (1989). Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J Biol Chem *264*, 7768-7771.

Heidemann, J., Maaser, C., Lugering, A., Spahn, T. W., Zimmer, K. P., Herbst, H., Rafiee, P., Domschke, W., Krieglstein, C. F., Binion, D. G., and Kucharzik, T. F. (2006). Expression of vascular cell adhesion molecule-1 (CD 106) in normal and neoplastic human esophageal squamous epithelium. Int J Oncol *28*, 77-85.

Heller, R., Unbehaun, A., Schellenberg, B., Mayer, B., Werner-Felmayer, G., and Werner, E. R. (2001). L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. J Biol Chem *276*, 40-47.

Henderson, R. B., Lim, L. H., Tessier, P. A., Gavins, F. N., Mathies, M., Perretti, M., and Hogg, N. (2001). The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. J Exp Med *194*, 219-226.

Herrmann, W., Obeid, R., and Jouma, M. (2003). Hyperhomocysteinemia and vitamin B-12 deficiency are more striking in Syrians than in Germans--causes and implications. Atherosclerosis *166*, 143-150.

Heydrick, S. J., Weiss, N., Thomas, S. R., Cap, A. P., Pimentel, D. R., Loscalzo, J., and Keaney, J. F., Jr. (2004). L-Homocysteine and L-homocystine stereospecifically induce endothelial nitric oxide synthase-dependent lipid peroxidation in endothelial cells. Free Radic Biol Med *36*, 632-640.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev *7*, 2135-2148.

Highsmith, R. F. (1998). Endothelin : molecular biology, physiology, and pathology (Totowa, N.J: Humana Press).

Hladovec, J., Sommerova, Z., and Pisarikova, A. (1997). Homocysteinemia and endothelial damage after methionine load. Thromb Res *88*, 361-364.

Hoefen, R. J., and Berk, B. C. (2002). The role of MAP kinases in endothelial activation. Vascul Pharmacol *38*, 271-273.

Hoffmann, J., Dimmeler, S., and Haendeler, J. (2003). Shear stress increases the amount of S-nitrosylated molecules in endothelial cells: important role for signal transduction. FEBS Lett *551*, 153-158.

Hofmann, M. A., Lalla, E., Lu, Y., Gleason, M. R., Wolf, B. M., Tanji, N., Ferran, L. J., Jr., Kohl, B., Rao, V., Kisiel, W., *et al.* (2001). Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. J Clin Invest *107*, 675-683.

Hoshino, S., Yoshida, M., Inoue, K., Yano, Y., Yanagita, M., Mawatari, H., Yamane, H., Kijima, T., Kumagai, T., Osaki, T., *et al.* (2005). Cigarette smoke extract induces endothelial cell injury via JNK pathway. Biochem Biophys Res Commun *329*, 58-63.

Huang, C. Y., and Ferrell, J. E., Jr. (1996). Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A *93*, 10078-10083.

Hurlimann, D., Forster, A., Noll, G., Enseleit, F., Chenevard, R., Distler, O., Bechir, M., Spieker, L. E., Neidhart, M., Michel, B. A., *et al.* (2002). Anti-tumor necrosis factor-alpha treatment improves endothelial function in patients with rheumatoid arthritis. Circulation *106*, 2184-2187.

Ide, T., Tsutsui, H., Hayashidani, S., Kang, D., Suematsu, N., Nakamura, K., Utsumi, H., Hamasaki, N., and Takeshita, A. (2001). Mitochondrial DNA damage and dysfunction

associated with oxidative stress in failing hearts after myocardial infarction. Circ Res *88*, 529-535.

Ignarro, L. J., Bush, P. A., Buga, G. M., Wood, K. S., Fukuto, J. M., and Rajfer, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem Biophys Res Commun *170*, 843-850.

Ip, Y. T., and Davis, R. J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol *10*, 205-219.

Jacobsen, D. W. (2000). Hyperhomocysteinemia and oxidative stress: time for a reality check? Arterioscler Thromb Vasc Biol *20*, 1182-1184.

Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., and Snyder, S. H. (2001). Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol *3*, 193-197.

Jakubowski, H. (2004). Molecular basis of homocysteine toxicity in humans. Cell Mol Life Sci *61*, 470-487.

Jakubowski, H., Zhang, L., Bardeguez, A., and Aviv, A. (2000). Homocysteine thiolactone and protein homocysteinylation in human endothelial cells: implications for atherosclerosis. Circ Res *87*, 45-51.

Ji, C., and Kaplowitz, N. (2004). Hyperhomocysteinemia, endoplasmic reticulum stress, and alcoholic liver injury. World J Gastroenterol *10*, 1699-1708.

Jia, E. Z., Yang, Z. J., Yuan, B., Zang, X. L., Wang, R. H., Zhu, T. B., Wang, L. S., Chen, B., and Ma, W. Z. (2005). Relationship between leukocyte count and angiographical characteristics of coronary atherosclerosis. Acta Pharmacol Sin *26*, 1057-1062.

Jin, L., Caldwell, R. B., Li-Masters, T., and Caldwell, R. W. (2007). Homocysteine induces endothelial dysfunction via inhibition of arginine transport. J Physiol Pharmacol *58*, 191-206.

Jones, D. A., Smith, C. W., Picker, L. J., and McIntire, L. V. (1996). Neutrophil adhesion to 24-hour IL-1-stimulated endothelial cells under flow conditions. J Immunol *157*, 858-863.

Jones, D. P., Eklöw, L., Thor, H., and Orrenius, S. (1981). Metabolism of hydrogen peroxide in isolated hepatocytes: Relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H2O2. Archives of Biochemistry and Biophysics *210*, 505-516.

Joosten, E., van den Berg, A., Riezler, R., Naurath, H. J., Lindenbaum, J., Stabler, S. P., and Allen, R. H. (1993). Metabolic evidence that deficiencies of vitamin B-12 (cobalamin), folate, and vitamin B-6 occur commonly in elderly people. Am J Clin Nutr *58*, 468-476.

Jousilahti, P., Vartiainen, E., Tuomilehto, J., and Puska, P. (1999). Sex, age, cardiovascular risk factors, and coronary heart disease: a prospective follow-up study of 14 786 middle-aged men and women in Finland. Circulation *99*, 1165-1172.

Juliano, R. L., and Haskill, S. (1993). Signal transduction from the extracellular matrix. J Cell Biol *120*, 577-585.

Jung, U., and Ley, K. (1999). Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. J Immunol *162*, 6755-6762.

Jung, U., Norman, K. E., Scharffetter-Kochanek, K., Beaudet, A. L., and Ley, K. (1998). Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. J Clin Invest *102*, 1526-1533.

Kallunki, T., Su, B., Tsigelny, I., Sluss, H. K., Derijard, B., Moore, G., Davis, R., and Karin, M. (1994). JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. Genes Dev *8*, 2996-3007.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell *120*, 649-661.

Kanani, P. M., Sinkey, C. A., Browning, R. L., Allaman, M., Knapp, H. R., and Haynes, W. G. (1999). Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. Circulation *100*, 1161-1168.

Kang, S. S., Zhou, J., Wong, P. W., Kowalisyn, J., and Strokosch, G. (1988). Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase. Am J Hum Genet *43*, 414-421.

Kaur, J., Woodman, R. C., and Kubes, P. (2003). P38 MAPK: critical molecule in thrombin-induced NF-kappa B-dependent leukocyte recruitment. Am J Physiol Heart Circ Physiol *284*, H1095-1103.

Kaushal, S., Amiel, G. E., Guleserian, K. J., Shapira, O. M., Perry, T., Sutherland, F. W., Rabkin, E., Moran, A. M., Schoen, F. J., Atala, A., *et al.* (2001). Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med *7*, 1035-1040.

Kelner, M. J., and Bagnell, R. (1990). Alteration of endogenous glutathione peroxidase, manganese superoxide dismutase, and glutathione transferase activity in cells transfected with a copper-zinc superoxide dismutase expression vector. Explanation for variations in paraquat resistance. J Biol Chem *265*, 10872-10875.

Kerkeni, M., Tnani, M., Chuniaud, L., Miled, A., Maaroufi, K., and Trivin, F. (2006). Comparative study on in vitro effects of homocysteine thiolactone and homocysteine on HUVEC cells: evidence for a stronger proapoptotic and proinflammative homocysteine thiolactone. Mol Cell Biochem *291*, 119-126.

Khan, B. V., Harrison, D. G., Olbrych, M. T., Alexander, R. W., and Medford, R. M. (1996). Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. Proc Natl Acad Sci U S A *93*, 9114-9119.

Khew-Goodall, Y., Wadham, C., Stein, B. N., Gamble, J. R., and Vadas, M. A. (1999). Stat6 activation is essential for interleukin-4 induction of P-selectin transcription in human umbilical vein endothelial cells. Arterioscler Thromb Vasc Biol *19*, 1421-1429.

Klatt, P., Molina, E. P., and Lamas, S. (1999). Nitric oxide inhibits c-Jun DNA binding by specifically targeted S-glutathionylation. J Biol Chem *274*, 15857-15864.

Kleemann, R., Zadelaar, S., and Kooistra, T. (2008). Cytokines and atherosclerosis: a comprehensive review of studies in mice. Cardiovasc Res *79*, 360-376.

Koga, T., Claycombe, K., and Meydani, M. (2002). Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells. Atherosclerosis *161*, 365-374.

Kokame, K., Kato, H., and Miyata, T. (1996). Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis. GRP78/BiP and novel genes. J Biol Chem *271*, 29659-29665.

Konstantopoulos, K., and McIntire, L. V. (1996). Effects of fluid dynamic forces on vascular cell adhesion. J Clin Invest *98*, 2661-2665.

Kosonen, O., Kankaanranta, H., Malo-Ranta, U., and Moilanen, E. (1999). Nitric oxide-releasing compounds inhibit neutrophil adhesion to endothelial cells. Eur J Pharmacol *382*, 111-117.

Kosonen, O., Kankaanranta, H., Uotila, J., and Moilanen, E. (2000). Inhibition by nitric oxide-releasing compounds of E-selectin expression in and neutrophil adhesion to human endothelial cells. Eur J Pharmacol *394*, 149-156.

Krause, A., Holtmann, H., Eickemeier, S., Winzen, R., Szamel, M., Resch, K., Saklatvala, J., and Kracht, M. (1998). Stress-activated protein kinase/Jun N-terminal kinase is required for interleukin (IL)-1-induced IL-6 and IL-8 gene expression in the human epidermal carcinoma cell line KB. J Biol Chem *273*, 23681-23689.

Kroll, J., and Waltenberger, J. (1998). VEGF-A Induces Expression of eNOS and iNOS in Endothelial Cells via VEGF Receptor-2 (KDR). Biochemical and Biophysical Research Communications *252*, 743-746.

Kubes, P., Jutila, M., and Payne, D. (1995). Therapeutic potential of inhibiting leukocyte rolling in ischemia/reperfusion. J Clin Invest *95*, 2510-2519.

Kubes, P., and Kanwar, S. (1994). Histamine induces leukocyte rolling in post-capillary venules. A P-selectin-mediated event. J Immunol *152*, 3570-3577.

Kubes, P., Suzuki, M., and Granger, D. N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A *88*, 4651-4655.

Kunkel, E. J., Dunne, J. L., and Ley, K. (2000). Leukocyte arrest during cytokine-dependent inflammation in vivo. J Immunol *164*, 3301-3308.

Kunsch, C., and Medford, R. M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. Circ Res *85*, 753-766.

Kuzkaya, N., Weissmann, N., Harrison, D. G., and Dikalov, S. (2003). Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem *278*, 22546-22554.

Kyaw, M., Yoshizumi, M., Tsuchiya, K., Izawa, Y., Kanematsu, Y., and Tamaki, T. (2004). Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases. Acta Pharmacol Sin *25*, 977-985.

Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. Nature *369*, 156-160.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lakshminarayanan, V., Beno, D. W. A., Costa, R. H., and Roebuck, K. A. (1997). Differential Regulation of Interleukin-8 and Intercellular Adhesion Molecule-1 by H2O2 and Tumor Necrosis Factor-alpha in Endothelial and Epithelial Cells. J Biol Chem *272*, 32910-32918.

Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E., and Harrison, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest *111*, 1201-1209.

Lawler, S., Fleming, Y., Goedert, M., and Cohen, P. (1998). Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. Curr Biol *8*, 1387-1390.

Lawrence de Koning, A. B., Werstuck, G. H., Zhou, J., and Austin, R. C. (2003). Hyperhomocysteinemia and its role in the development of atherosclerosis. Clin Biochem *36*, 431-441.

Lawrence, M. B., Bainton, D. F., and Springer, T. A. (1994). Neutrophil tethering to and rolling on E-selectin are separable by requirement for L-selectin. Immunity *1*, 137-145.

Lawrence, M. B., McIntire, L. V., and Eskin, S. G. (1987). Effect of flow on polymorphonuclear leukocyte/endothelial cell adhesion. Blood *70*, 1284-1290.

Lawrence, M. B., and Springer, T. A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859-873.

Lee, H. Y., Chae, I. H., Kim, H. S., Park, Y. B., Choi, Y. S., Lee, Y. W., Park, S. J., and Cha, Y. J. (2002). Differential effects of homocysteine on porcine endothelial and vascular smooth muscle cells. J Cardiovasc Pharmacol *39*, 643-651.

Lee, S. J., Kim, K. M., Namkoong, S., Kim, C. K., Kang, Y. C., Lee, H., Ha, K. S., Han, J. A., Chung, H. T., Kwon, Y. G., and Kim, Y. M. (2005). Nitric oxide inhibition of homocysteine-induced human endothelial cell apoptosis by down-regulation of p53-dependent Noxa expression through the formation of S-nitrosohomocysteine. J Biol Chem *280*, 5781-5788.

Lefer, A. M. (1997). Nitric oxide: nature's naturally occurring leukocyte inhibitor. Circulation *95*, 553-554.

Lentz, S. R. (2005). Mechanisms of homocysteine-induced atherothrombosis. J Thromb Haemost *3*, 1646-1654.

Lentz, S. R., and Haynes, W. G. (2004). Homocysteine: is it a clinically important cardiovascular risk factor? Cleve Clin J Med *71*, 729-734.

Lentz, S. R., Sobey, C. G., Piegors, D. J., Bhopatkar, M. Y., Faraci, F. M., Malinow, M. R., and Heistad, D. D. (1996). Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. J Clin Invest *98*, 24-29.

Lerman, A., and Burnett, J. C., Jr. (1992). Intact and altered endothelium in regulation of vasomotion. Circulation *86*, III12-19.

Levrand, S., Pacher, P., Pesse, B., Rolli, J., Feihl, F., Waeber, B., and Liaudet, L. (2007). Homocysteine induces cell death in H9C2 cardiomyocytes through the generation of peroxynitrite. Biochem Biophys Res Commun *359*, 445-450.

Ley, K. (2003). The role of selectins in inflammation and disease. Trends Mol Med *9*, 263-268.

Libby, P., Ordovas, J. M., Auger, K. R., Robbins, A. H., Birinyi, L. K., and Dinarello, C. A. (1986). Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. Am J Pathol *124*, 179-185.

Lim, S., Kim, M. S., Park, K. S., Lee, J. H., An, G. H., Yim, M. J., Song, J., Pak, Y. K., and Lee, H. K. (2001). Correlation of plasma homocysteine and mitochondrial DNA content in peripheral blood in healthy women. Atherosclerosis *158*, 399-405.

Lim, Y.-C. (1995) The development of an in-vitro model of reperfusion injury : investigation in-vitro of human saphenous vein and dermal microvascular endothelial cell responses to hypoxia-reoxygenation and a comparison of these responses to those of cells stimulated with inflammatory mediators, Ph.D. thesis. Typescript., Bradford,.

Lin, C. P., Chen, Y. H., Lin, W. T., Leu, H. B., Liu, T. Z., Huang, S. L., and Chen, J. W. (2008). Direct effect of statins on homocysteine-induced endothelial adhesiveness: potential impact to human atherosclerosis. Eur J Clin Invest *38*, 106-116.

Lincoln, J., Hoyle, C. H. V., and Burnstock, G. (1997). Nitric oxide in health and disease (Cambridge: Cambridge University Press).

Ling, Q., and Hajjar, K. A. (2000). Inhibition of endothelial cell thromboresistance by homocysteine. J Nutr *130*, 373S-376S.

Lonn, E., Yusuf, S., Arnold, M. J., Sheridan, P., Pogue, J., Micks, M., McQueen, M. J., Probstfield, J., Fodor, G., Held, C., and Genest, J., Jr. (2006). Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med *354*, 1567-1577.

Lorant, D. E., Topham, M. K., Whatley, R. E., McEver, R. P., McIntyre, T. M., Prescott, S. M., and Zimmerman, G. A. (1993). Inflammatory roles of P-selectin. J Clin Invest *92*, 559-570.

Loscalzo, J. (1996). The oxidant stress of hyperhomocyst(e)inemia. J Clin Invest *98*, 5-7.

Loscalzo, J. (2006). Homocysteine trials--clear outcomes for complex reasons. N Engl J Med *354*, 1629-1632.

Lowenstein, C. J. (2007). Nitric oxide regulation of protein trafficking in the cardiovascular system. Cardiovasc Res *75*, 240-246.

Lowenstein, C. J., Morrell, C. N., and Yamakuchi, M. (2005). Regulation of Weibel-Palade body exocytosis. Trends Cardiovasc Med *15*, 302-308.

Lu, S. C. (2000). S-Adenosylmethionine. Int J Biochem Cell Biol *32*, 391-395.

Luscinskas, F. W., Ding, H., and Lichtman, A. H. (1995). P-selectin and vascular cell adhesion molecule 1 mediate rolling and arrest, respectively, of CD4+ T lymphocytes on tumor necrosis factor alpha-activated vascular endothelium under flow. J Exp Med *181*, 1179-1186.

Luscinskas, F. W., and Lawler, J. (1994). Integrins as dynamic regulators of vascular function. Faseb J *8*, 929-938.

Lusis, A. J. (2000). Atherosclerosis. Nature *407*, 233-241.

Mann, G. E., Yudilevich, D. L., and Sobrevia, L. (2003). Regulation of Amino Acid and Glucose Transporters in Endothelial and Smooth Muscle Cells. Physiol Rev *83*, 183-252.

Mansoor, M. A., Seljeflot, I., Arnesen, H., Knudsen, A., Bates, C. J., Mishra, G., and Larsen, T. W. (2004). Endothelial cell adhesion molecules in healthy adults during acute hyperhomocysteinemia and mild hypertriglyceridemia. Clin Biochem *37*, 408-414.

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev *4*, 82-89.

Marshall, H. E., and Stamler, J. S. (2001). Inhibition of NF-kappa B by S-nitrosylation. Biochemistry *40*, 1688-1693.

Marshall, L. J., Ramdin, L. S., Brooks, T., PC, D. P., and Shute, J. K. (2003). Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. J Immunol *171*, 2057-2065.

Mato, J. M., Corrales, F. J., Lu, S. C., and Avila, M. A. (2002). S-Adenosylmethionine: a control switch that regulates liver function. Faseb J *16*, 15-26.

Matsushita, K., Morrell, C. N., Cambien, B., Yang, S. X., Yamakuchi, M., Bao, C., Hara, M. R., Quick, R. A., Cao, W., O'Rourke, B., *et al.* (2003). Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell *115*, 139-150.

Mayadas, T. N., Johnson, R. C., Rayburn, H., Hynes, R. O., and Wagner, D. D. (1993). Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. Cell *74*, 541-554.

McCully, K. S. (1969). Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol *56*, 111-128.

McCully, K. S., and Wilson, R. B. (1975). Homocysteine theory of arteriosclerosis. Atherosclerosis *22*, 215-227.

McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L., and Bainton, D. F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies, In J Clin Invest, pp. 92-99.

McEver, R. P., and Cummings, R. D. (1997). Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. J Clin Invest *100*, 485-491.

Medina, M., Urdiales, J. L., and Amores-Sanchez, M. I. (2001). Roles of homocysteine in cell metabolism: old and new functions. Eur J Biochem *268*, 3871-3882.

Mensah, G. A., Ryan, U. S., Hooper, W. C., Engelgau, M. M., Callow, A. D., Kapuku, G. K., and Mantovani, A. (2007). Vascular endothelium summary statement II: Cardiovascular disease prevention and control. Vascul Pharmacol *46*, 318-320.

Metzler, B., Hu, Y., Dietrich, H., and Xu, Q. (2000). Increased expression and activation of stress-activated protein kinases/c-Jun NH(2)-terminal protein kinases in atherosclerotic lesions coincide with p53. Am J Pathol *156*, 1875-1886.

Meyer, M., Pahl, H. L., and Baeuerle, P. A. (1994). Regulation of the transcription factors NF-kappa B and AP-1 by redox changes. Chem Biol Interact *91*, 91-100.

Miho, N., Ishida, T., Kuwaba, N., Ishida, M., Shimote-Abe, K., Tabuchi, K., Oshima, T., Yoshizumi, M., and Chayama, K. (2005). Role of the JNK pathway in thrombin-induced ICAM-1 expression in endothelial cells. Cardiovasc Res *68*, 289-298.

Min, W., and Pober, J. S. (1997). TNF initiates E-selectin transcription in human endothelial cells through parallel TRAF-NF-kappa B and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 pathways. J Immunol *159*, 3508-3518.

Misra, H. P. (1974). Generation of superoxide free radical during the autoxidation of thiols. J Biol Chem *249*, 2151-2155.

Morrell, C. N., Matsushita, K., Chiles, K., Scharpf, R. B., Yamakuchi, M., Mason, R. J., Bergmeier, W., Mankowski, J. L., Baldwin, W. M., 3rd, Faraday, N., and Lowenstein, C. J. (2005). Regulation of platelet granule exocytosis by S-nitrosylation. Proc Natl Acad Sci U S A *102*, 3782-3787.

Muller, W. A. (2002). Leukocyte-endothelial cell interactions in the inflammatory response. Lab Invest *82*, 521-533.

Nagai, Y., Tasaki, H., Takatsu, H., Nihei, S., Yamashita, K., Toyokawa, T., and Nakashima, Y. (2001). Homocysteine inhibits angiogenesis in vitro and in vivo. Biochem Biophys Res Commun *281*, 726-731.

Nappo, F., De Rosa, N., Marfella, R., De Lucia, D., Ingrosso, D., Perna, A. F., Farzati, B., and Giugliano, D. (1999). Impairment of endothelial functions by acute hyperhomocysteinemia and reversal by antioxidant vitamins. Jama *281*, 2113-2118.

Naseem, K. M. (2005). The role of nitric oxide in cardiovascular diseases. Mol Aspects Med *26*, 33-65.

Natarajan, R., Gupta, S., Fisher, B. J., Ghosh, S., and Fowler, A. A., 3rd (2001). Nitric oxide suppresses IL-8 transcription by inhibiting c-Jun N-terminal kinase-induced AP-1 activation. Exp Cell Res *266*, 203-212.

Nicolaou, A., Ast, T., Anderson, M. M., Schmidt, W., Yeboah, F. A., and Gibbons, W. A. (1994a). Role of vitamin B12 enzymes in platelet cell signalling, adhesion and aggregation. Biochem Soc Trans *22*, 224S.

Nicolaou, A., Ast, T., Garcia, C. V., Anderson, M. M., Gibbons, J. M., and Gibbons, W. A. (1994b). In vitro NO and N2O inhibition of the branch point enzyme vitamin B12 dependent methionine synthase from rat brain synaptosomes. Biochem Soc Trans *22*, 296S.

Nicolaou, A., Waterfield, C. J., Kenyon, S. H., and Gibbons, W. A. (1997). The inactivation of methionine synthase in isolated rat hepatocytes by sodium nitroprusside. Eur J Biochem *244*, 876-882.

Nikitina, E. Y., Freidlin, I. S., Churakov, G. A., Vinogradov, A. G., Pigarevsky, P. V., Nagomev, V. A., and Denisenko, A. D. (1997). Tumour necrosis factor-[alpha] (TNF) production in atherogenesis. Immunology Letters *56*, 282-283.

Nishio, H., Matsui, K., Tsuji, H., Tamura, A., and Suzuki, K. (2001). Immunohistochemical study of the phosphorylated and activated form of c-Jun NH2-terminal kinase in human aorta. Histochem J *33*, 167-171.

Nordberg, J., and Arner, E. S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med *31*, 1287-1312.

Nygard, O., Refsum, H., Ueland, P. M., and Vollset, S. E. (1998). Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr *67*, 263-270.

Ohashi, R., Yan, S., Mu, H., Chai, H., Yao, Q., Lin, P. H., and Chen, C. (2006). Effects of homocysteine and ginsenoside Rb1 on endothelial proliferation and superoxide anion production. J Surg Res *133*, 89-94.

Ohta, H., Wada, H., Niwa, T., Kirii, H., Iwamoto, N., Fujii, H., Saito, K., Sekikawa, K., and Seishima, M. (2005). Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. Atherosclerosis *180*, 11-17.

Ohyama, C., Tsuboi, S., and Fukuda, M. (1999). Dual roles of sialyl Lewis X oligosaccharides in tumor metastasis and rejection by natural killer cells. Embo J *18*, 1516-1525.

Outinen, P. A., Sood, S. K., Liaw, P. C., Sarge, K. D., Maeda, N., Hirsh, J., Ribau, J., Podor, T. J., Weitz, J. I., and Austin, R. C. (1998). Characterization of the stress-inducing effects of homocysteine. Biochem J *332 (Pt 1)*, 213-221.

Outinen, P. A., Sood, S. K., Pfeifer, S. I., Pamidi, S., Podor, T. J., Li, J., Weitz, J. I., and Austin, R. C. (1999). Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. Blood *94*, 959-967.

Palacios, M., Knowles, R. G., Palmer, R. M., and Moncada, S. (1989). Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem Biophys Res Commun *165*, 802-809.

Palmer, R. M., and Moncada, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. Biochem Biophys Res Commun *158*, 348-352.

Paolini, M., Sapone, A., Canistro, D., Chieco, P., and Valgimigli, L. (2003). Antioxidant vitamins for prevention of cardiovascular disease. Lancet *362*, 920; author reply 921.

Papatheodorou, L., and Weiss, N. (2007). Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal *9*, 1941-1958.

Park, H. S., Huh, S. H., Kim, M. S., Kim, D. Y., Gwag, B. J., Cho, S. G., and Choi, E. J. (2006). Neuronal nitric oxide synthase (nNOS) modulates the JNK1 activity through redox mechanism: a cGMP independent pathway. Biochem Biophys Res Commun *346*, 408-414.

Park, H. S., Huh, S. H., Kim, M. S., Lee, S. H., and Choi, E. J. (2000a). Nitric oxide negatively regulates c-Jun N-terminal kinase/stress-activated protein kinase by means of S-nitrosylation. Proc Natl Acad Sci U S A *97*, 14382-14387.

Park, H. S., Park, E., Kim, M. S., Ahn, K., Kim, I. Y., and Choi, E. J. (2000b). Selenite inhibits the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) through a thiol redox mechanism. J Biol Chem *275*, 2527-2531.

Park, J. B. (2001). Reduction of dehydroascorbic acid by homocysteine. Biochim Biophys Acta *1525*, 173-179.

Patterson, C., Ruef, J., Madamanchi, N. R., Barry-Lane, P., Hu, Z., Horaist, C., Ballinger, C. A., Brasier, A. R., Bode, C., and Runge, M. S. (1999). Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin. Evidence that p47(phox) may participate in forming this oxidase in vitro and in vivo. J Biol Chem *274*, 19814-19822.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev *22*, 153-183.

Penberthy, T. W., Jiang, Y., and Graves, D. T. (1997). Leukocyte adhesion molecules. Crit Rev Oral Biol Med *8*, 380-388.

Peracchi, M., Bamonti Catena, F., Pomati, M., De Franceschi, M., and Scalabrino, G. (2001). Human cobalamin deficiency: alterations in serum tumour necrosis factor-alpha and epidermal growth factor. Eur J Haematol *67*, 123-127.

Pietersma, A., Tilly, B. C., Gaestel, M., de Jong, N., Lee, J. C., Koster, J. F., and Sluiter, W. (1997). P38 Mitogen Activated Protein Kinase Regulates Endothelial VCAM-1 Expression at the Post-transcriptional Level. Biochemical and Biophysical Research Communications *230*, 44-48.

Poddar, R., Sivasubramanian, N., DiBello, P. M., Robinson, K., and Jacobsen, D. W. (2001). Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease. Circulation *103*, 2717-2723.

Postea, O., Krotz, F., Henger, A., Keller, C., and Weiss, N. (2006). Stereospecific and redox-sensitive increase in monocyte adhesion to endothelial cells by homocysteine. Arterioscler Thromb Vasc Biol *26*, 508-513.

Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H., and Rosen, G. M. (1992). Generation of superoxide by purified brain nitric oxide synthase. J Biol Chem *267*, 24173-24176.

Proud, C. G. (1994). Peptide-chain elongation in eukaryotes. Mol Biol Rep *19*, 161-170.

Pruefer, D., Scalia, R., and Lefer, A. M. (1999). Homocysteine provokes leukocyte-endothelium interaction by downregulation of nitric oxide. Gen Pharmacol *33*, 487-498.

Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991). Phosphorylation of c-jun mediated by MAP kinases. Nature *353*, 670-674.

Raitano, A. B., Halpern, J. R., Hambuch, T. M., and Sawyers, C. L. (1995). The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. Proc Natl Acad Sci U S A *92*, 11746-11750.

Ralph, J. A., and Morand, E. F. (2008). MAPK phosphatases as novel targets for rheumatoid arthritis. Expert Opin Ther Targets *12*, 795-808.

Rattan, A. K., and Arad, Y. (1998). Temporal and kinetic determinants of the inhibition of LDL oxidation by N-acetylcysteine (NAC). Atherosclerosis *138*, 319-327.

Read, M. A., Whitley, M. Z., Gupta, S., Pierce, J. W., Best, J., Davis, R. J., and Collins, T. (1997). Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J Biol Chem *272*, 2753-2761.

Reape, T. J., and Groot, P. H. E. (1999). Chemokines and atherosclerosis. Atherosclerosis *147*, 213-225.

Reinhard, C., Shamoon, B., Shyamala, V., and Williams, L. T. (1997). Tumor necrosis factor alpha-induced activation of c-jun N-terminal kinase is mediated by TRAF2. Embo J *16*, 1080-1092.

Ricci, R., Sumara, G., Sumara, I., Rozenberg, I., Kurrer, M., Akhmedov, A., Hersberger, M., Eriksson, U., Eberli, F. R., Becher, B., *et al.* (2004). Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. Science *306*, 1558-1561.

Rodgers, G. M., and Kane, W. H. (1986). Activation of endogenous factor V by a homocysteine-induced vascular endothelial cell activator. J Clin Invest *77*, 1909-1916.

Rodríguez-Nieto, S., Chavarría, T., Martínez-Poveda, B., Sánchez-Jiménez, F., Rodríguez Quesada, A., and Medina, M. Á. (2002). Anti-angiogenic effects of homocysteine on cultured endothelial cells. Biochemical and Biophysical Research Communications *293*, 497-500.

Roebuck, K. A. (1999). Regulation of interleukin-8 gene expression. J Interferon Cytokine Res *19*, 429-438.

Rosen, G. M., and Freeman, B. A. (1984). Detection of superoxide generated by endothelial cells. Proc Natl Acad Sci U S A *81*, 7269-7273.

Rot, A. (1992). Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. Immunol Today *13*, 291-294.

Roth, J., Goebeler, M., Ludwig, S., Wagner, L., Kilian, K., Sorg, C., Harms, E., Schulze-Osthoff, K., and Koch, H. (2001). Homocysteine inhibits tumor necrosis factor-induced activation of endothelium via modulation of nuclear factor-kappa b activity. Biochim Biophys Acta *1540*, 154-165.

Roulston, A., Reinhard, C., Amiri, P., and Williams, L. T. (1998). Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. J Biol Chem *273*, 10232-10239.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell *78*, 1027-1037.

Royall, J. A., and Ischiropoulos, H. (1993). Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch Biochem Biophys *302*, 348-355.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., and Freeman, B. A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. J Biol Chem *269*, 26066-26075.

Ruoslahti, E. (1991). Integrins. J Clin Invest *87*, 1-5.

Rus, H. G., Vlaicu, R., and Niculescu, F. (1996). Interleukin-6 and interleukin-8 protein and gene expression in human arterial atherosclerotic wall. Atherosclerosis *127*, 263-271.

Ryu, S., Huppmann, A. R., Sambangi, N., Takacs, P., and Kauma, S. W. (2007). Increased leukocyte adhesion to vascular endothelium in preeclampsia is inhibited by antioxidants. Am J Obstet Gynecol *196*, 400 e401-407; discussion 400 e407-408.

Sabapathy, K., Hochedlinger, K., Nam, S. Y., Bauer, A., Karin, M., and Wagner, E. F. (2004). Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. Mol Cell *15*, 713-725.

Sako, D., Comess, K. M., Barone, K. M., Camphausen, R. T., Cumming, D. A., and Shaw, G. D. (1995). A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell *83*, 323-331.

Sato, M., Miyazaki, T., Nagaya, T., Murata, Y., Ida, N., Maeda, K., and Seo, H. (1996). Antioxidants inhibit tumor necrosis factor-alpha mediated stimulation of interleukin-8, monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. J Rheumatol *23*, 432-438.

Sattar, N., Crompton, P., Cherry, L., Kane, D., Lowe, G., and McInnes, I. B. (2007). Effects of tumor necrosis factor blockade on cardiovascular risk factors in psoriatic arthritis: a double-blind, placebo-controlled study. Arthritis Rheum *56*, 831-839.

Sawle, P., Foresti, R., Green, C. J., and Motterlini, R. (2001). Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia. FEBS Lett *508*, 403-406.

Scalera, F., Martens-Lobenhoffer, J., Tager, M., Bukowska, A., Lendeckel, U., and Bode-Boger, S. M. (2006). Effect of L-arginine on asymmetric dimethylarginine (ADMA) or homocysteine-accelerated endothelial cell aging. Biochem Biophys Res Commun *345*, 1075-1082.

Schachinger, V., Britten, M. B., and Zeiher, A. M. (2000). Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation *101*, 1899-1906.

Schlossmann, J., Feil, R., and Hofmann, F. (2003). Signaling through NO and cGMP-dependent protein kinases. Ann Med *35*, 21-27.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., and Mayer, B. (1996). Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. Mol Pharmacol *50*, 1-5.

Schreyer, S. A., Peschon, J. J., and LeBoeuf, R. C. (1996). Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem *271*, 26174-26178.

Seeliger, M. A., and Kuriyan, J. (2009). A MAPK scaffold lends a helping hand. Cell *136*, 994-996.

Selhub, J. (1999). Homocysteine metabolism. Annu Rev Nutr *19*, 217-246.

Selhub, J., Jacques, P. F., Bostom, A. G., D'Agostino, R. B., Wilson, P. W., Belanger, A. J., O'Leary, D. H., Wolf, P. A., Schaefer, E. J., and Rosenberg, I. H. (1995). Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. N Engl J Med *332*, 286-291.

Selhub, J., Jacques, P. F., Rosenberg, I. H., Rogers, G., Bowman, B. A., Gunter, E. W., Wright, J. D., and Johnson, C. L. (1999). Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991-1994): population reference ranges and contribution of vitamin status to high serum concentrations. Ann Intern Med *131*, 331-339.

Selhub, J., and Miller, J. W. (1992). The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. Am J Clin Nutr *55*, 131-138.

Sengupta, S., Wehbe, C., Majors, A. K., Ketterer, M. E., DiBello, P. M., and Jacobsen, D. W. (2001). Relative roles of albumin and ceruloplasmin in the formation of homocystine, homocysteine-cysteine-mixed disulfide, and cystine in circulation. J Biol Chem *276*, 46896-46904.

Settergren, M., and Tornvall, P. (2004). Does TNF-alpha blockade cause plaque rupture? Atherosclerosis *173*, 149.

Shamu, C. E., and Walter, P. (1996). Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. Embo J *15*, 3028-3039.

Sheikh, S., Rainger, G. E., Gale, Z., Rahman, M., and Nash, G. B. (2003). Exposure to fluid shear stress modulates the ability of endothelial cells to recruit neutrophils in response to tumor necrosis factor-alpha: a basis for local variations in vascular sensitivity to inflammation. Blood *102*, 2828-2834.

Shen, H. M., and Liu, Z. G. (2006). JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. Free Radic Biol Med *40*, 928-939.

Shigeta, A., Matsumoto, M., Tedder, T. F., Lowe, J. B., Miyasaka, M., and Hirata, T. (2008). An L-selectin ligand distinct from P-selectin glycoprotein ligand-1 is expressed on endothelial cells and promotes neutrophil rolling in inflammation. Blood *112*, 4915-4923.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. Exp Physiol *82*, 291-295.

Silverman, M. D., Tumuluri, R. J., Davis, M., Lopez, G., Rosenbaum, J. T., and Lelkes, P. I. (2002). Homocysteine upregulates vascular cell adhesion molecule-1 expression in cultured human aortic endothelial cells and enhances monocyte adhesion. Arterioscler Thromb Vasc Biol *22*, 587-592.

Siow, R. C. M., Sato, H., Leake, D. S., Pearson, J. D., Bannai, S., and Mann, G. E. (1998). Vitamin C Protects Human Arterial Smooth Muscle Cells Against Atherogenic Lipoproteins : Effects of Antioxidant Vitamins C and E on Oxidized LDL–Induced Adaptive Increases in Cystine Transport and Glutathione. Arterioscler Thromb Vasc Biol *18*, 1662-1670.

Siow, Y. L., Au-Yeung, K. K., Woo, C. W., and O, K. (2006). Homocysteine stimulates phosphorylation of NADPH oxidase p47phox and p67phox subunits in monocytes via protein kinase Cbeta activation. Biochem J *398*, 73-82.

Skrypina, N. A., Timofeeva, A. V., Khaspekov, G. L., Savochkina, L. P., and Beabealashvilli, R. (2003). Total RNA suitable for molecular biology analysis. J Biotechnol *105*, 1-9.

Sligh, J. E., Jr., Ballantyne, C. M., Rich, S. S., Hawkins, H. K., Smith, C. W., Bradley, A., and Beaudet, A. L. (1993). Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc Natl Acad Sci U S A *90*, 8529-8533.

Sluss, H. K., Barrett, T., Derijard, B., and Davis, R. J. (1994). Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol Cell Biol *14*, 8376-8384.

Sochman, J. (2002). N-acetylcysteine in acute cardiology: 10 years later: what do we know and what would we like to know?! J Am Coll Cardiol *39*, 1422-1428.

Sorescu, D., Weiss, D., Lassegue, B., Clempus, R. E., Szocs, K., Sorescu, G. P., Valppu, L., Quinn, M. T., Lambeth, J. D., Vega, J. D., *et al.* (2002). Superoxide Production and Expression of Nox Family Proteins in Human Atherosclerosis. Circulation *105*, 1429-1435.

Sotiriou, S. N., Orlova, V. V., Al-Fakhri, N., Ihanus, E., Economopoulou, M., Isermann, B., Bdeir, K., Nawroth, P. P., Preissner, K. T., Gahmberg, C. G., *et al.* (2006). Lipoprotein(a) in atherosclerotic plaques recruits inflammatory cells through interaction with Mac-1 integrin. Faseb J *20*, 559-561.

Sperandio, M., Smith, M. L., Forlow, S. B., Olson, T. S., Xia, L., McEver, R. P., and Ley, K. (2003). P-selectin glycoprotein ligand-1 mediates L-selectin-dependent leukocyte rolling in venules. J Exp Med *197*, 1355-1363.

Spiecker, M., Darius, H., Kaboth, K., Hubner, F., and Liao, J. K. (1998). Differential regulation of endothelial cell adhesion molecule expression by nitric oxide donors and antioxidants. J Leukoc Biol *63*, 732-739.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301-314.

Stamler, J. S., Osborne, J. A., Jaraki, O., Rabbani, L. E., Mullins, M., Singel, D., and Loscalzo, J. (1993). Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest *91*, 308-318.

Stampfer, M. J., Malinow, M. R., Willett, W. C., Newcomer, L. M., Upson, B., Ullmann, D., Tishler, P. V., and Hennekens, C. H. (1992). A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. Jama *268*, 877-881.

Stangl, V., Gunther, C., Jarrin, A., Bramlage, P., Moobed, M., Staudt, A., Baumann, G., Stangl, K., and Felix, S. B. (2001). Homocysteine inhibits TNF-alpha-induced endothelial adhesion molecule expression and monocyte adhesion via nuclear factor-kappaB dependent pathway. Biochem Biophys Res Commun *280*, 1093-1100.

Stanner, S. A., Hughes, J., Kelly, C. N., and Buttriss, J. (2004). A review of the epidemiological evidence for the 'antioxidant hypothesis'. Public Health Nutr *7*, 407-422.

Steeber, D. A., Campbell, M. A., Basit, A., Ley, K., and Tedder, T. F. (1998). Optimal selectin-mediated rolling of leukocytes during inflammation in vivo requires intercellular adhesion molecule-1 expression. Proc Natl Acad Sci U S A *95*, 7562-7567.

Stein, B., Yang, M. X., Young, D. B., Janknecht, R., Hunter, T., Murray, B. W., and Barbosa, M. S. (1997). p38-2, a novel mitogen-activated protein kinase with distinct properties. J Biol Chem *272*, 19509-19517.

Stewart, M., Thiel, M., and Hogg, N. (1995). Leukocyte integrins. Curr Opin Cell Biol *7*, 690-696.

Stuhlinger, M. C., Tsao, P. S., Her, J. H., Kimoto, M., Balint, R. F., and Cooke, J. P. (2001). Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. Circulation *104*, 2569-2575.

Su, L. C., Bui, M., Kardinaal, A., Gomez-Aracena, J., Martin-Moreno, J., Martin, B., Thamm, M., Simonsen, N., van't Veer, P., Kok, F., *et al.* (1998). Differences between plasma and adipose tissue biomarkers of carotenoids and tocopherols. Cancer Epidemiol Biomarkers Prev *7*, 1043-1048.

Su, S. J., Huang, L. W., Pai, L. S., Liu, H. W., and Chang, K. L. (2005). Homocysteine at pathophysiologic concentrations activates human monocyte and induces cytokine expression and inhibits macrophage migration inhibitory factor expression. Nutrition *21*, 994-1002.

Subramaniam, M., Koedam, J. A., and Wagner, D. D. (1993). Divergent fates of P- and E-selectins after their expression on the plasma membrane. Mol Biol Cell *4*, 791-801.

Sumara, G., Belwal, M., and Ricci, R. (2005). "Jnking" atherosclerosis. Cell Mol Life Sci *62*, 2487-2494.

Sun, J., Xin, C., Eu, J. P., Stamler, J. S., and Meissner, G. (2001). Cysteine-3635 is responsible for skeletal muscle ryanodine receptor modulation by NO. Proc Natl Acad Sci U S A *98*, 11158-11162.

Suzuki, Y. J., Lorenzi, M. V., Shi, S. S., Day, R. M., and Blumberg, J. B. (2000). Homocysteine exerts cell type-specific inhibition of AP-1 transcription factor. Free Radic Biol Med *28*, 39-45.

Szczepankiewicz, B. G., Kosogof, C., Nelson, L. T., Liu, G., Liu, B., Zhao, H., Serby, M. D., Xin, Z., Liu, M., Gum, R. J., *et al.* (2006). Aminopyridine-based c-Jun N-terminal kinase inhibitors with cellular activity and minimal cross-kinase activity. J Med Chem *49*, 3563-3580.

Taddei, A., Giampietro, C., Conti, A., Orsenigo, F., Breviario, F., Pirazzoli, V., Potente, M., Daly, C., Dimmeler, S., and Dejana, E. (2008). Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol *10*, 923-934.

Takacs, P., Kauma, S. W., Sholley, M. M., Walsh, S. W., Dinsmoor, M. J., and Green, K. (2001). Increased circulating lipid peroxides in severe preeclampsia activate NF-kappaB and upregulate ICAM-1 in vascular endothelial cells. Faseb J *15*, 279-281.

Takahashi, M., Ikeda, U., Masuyama, J., Funayama, H., Kano, S., and Shimada, K. (1996). Nitric oxide attenuates adhesion molecule expression in human endothelial cells. Cytokine *8*, 817-821.

Takaishi, H., Taniguchi, T., Takahashi, A., Ishikawa, Y., and Yokoyama, M. (2003). High glucose accelerates MCP-1 production via p38 MAPK in vascular endothelial cells. Biochemical and Biophysical Research Communications *305*, 122-128.

Tampo, Y., Kotamraju, S., Chitambar, C. R., Kalivendi, S. V., Keszler, A., Joseph, J., and Kalyanaraman, B. (2003). Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells: role of transferrin receptor-dependent iron uptake in apoptosis. Circ Res *92*, 56-63.

Tarpey, M. M., and Fridovich, I. (2001). Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. Circ Res *89*, 224-236.

Tedder, T. F., Isaacs, C. M., Ernst, T. J., Demetri, G. D., Adler, D. A., and Disteche, C. M. (1989). Isolation and chromosomal localization of cDNAs encoding a novel human lymphocyte cell surface molecule, LAM-1. Homology with the mouse lymphocyte homing receptor and other human adhesion proteins. J Exp Med *170*, 123-133.

Tedgui, A., and Mallat, Z. (2006). Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev *86*, 515-581.

Tomasian, D., Keaney, J. F., and Vita, J. A. (2000). Antioxidants and the bioactivity of endothelium-derived nitric oxide. Cardiovasc Res *47*, 426-435.

Toole, J. F., Malinow, M. R., Chambless, L. E., Spence, J. D., Pettigrew, L. C., Howard, V. J., Sides, E. G., Wang, C. H., and Stampfer, M. (2004). Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. Jama *291*, 565-575.

Topal, G., Brunet, A., Millanvoye, E., Boucher, J. L., Rendu, F., Devynck, M. A., and David-Dufilho, M. (2004). Homocysteine induces oxidative stress by uncoupling of NO

synthase activity through reduction of tetrahydrobiopterin. Free Radic Biol Med *36*, 1532-1541.

Tournier, C., Dong, C., Turner, T. K., Jones, S. N., Flavell, R. A., and Davis, R. J. (2001). MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. Genes Dev *15*, 1419-1426.

Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science *288*, 870-874.

Tsai, J. C., Perrella, M. A., Yoshizumi, M., Hsieh, C. M., Haber, E., Schlegel, R., and Lee, M. E. (1994). Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. Proc Natl Acad Sci U S A *91*, 6369-6373.

Tyagi, N., Ovechkin, A. V., Lominadze, D., Moshal, K. S., and Tyagi, S. C. (2006). Mitochondrial mechanism of microvascular endothelial cells apoptosis in hyperhomocysteinemia. J Cell Biochem *98*, 1150-1162.

Tyagi, N., Sedoris, K. C., Steed, M., Ovechkin, A. V., Moshal, K. S., and Tyagi, S. C. (2005). Mechanisms of homocysteine-induced oxidative stress. Am J Physiol Heart Circ Physiol *289*, H2649-2656.

Tyagi, S. C. (1998). Homocysteine redox receptor and regulation of extracellular matrix components in vascular cells. Am J Physiol *274*, C396-405.

Ullrich, V., and Bachschmid, M. (2000). Superoxide as a Messenger of Endothelial Function. Biochemical and Biophysical Research Communications *278*, 1-8.

Ungvari, Z., Csiszar, A., Edwards, J. G., Kaminski, P. M., Wolin, M. S., Kaley, G., and Koller, A. (2003). Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor-alpha, NAD(P)H oxidase, and inducible nitric oxide synthase. Arterioscler Thromb Vasc Biol *23*, 418-424.

Upchurch, G. R., Jr., Welch, G. N., Fabian, A. J., Freedman, J. E., Johnson, J. L., Keaney, J. F., Jr., and Loscalzo, J. (1997). Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem *272*, 17012-17017.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science *287*, 664-666.

Ushio-Fukai, M., Alexander, R. W., Akers, M., and Griendling, K. K. (1998). p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. J Biol Chem *273*, 15022-15029.

van Meurs, J. B., Dhonukshe-Rutten, R. A., Pluijm, S. M., van der Klift, M., de Jonge, R., Lindemans, J., de Groot, L. C., Hofman, A., Witteman, J. C., van Leeuwen, J. P., *et al.* (2004). Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med *350*, 2033-2041.

Ventura, P., Panini, R., Pasini, M. C., Scarpetta, G., and Salvioli, G. (1999). N -ACETYL-CYSTEINE REDUCES HOMOCYSTEINE PLASMA LEVELS AFTER SINGLE

INTRAVENOUS ADMINISTRATION BY INCREASING THIOLS URINARY EXCRETION. Pharmacological Research *40*, 345-350.

Vestweber, D., and Blanks, J. E. (1999). Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev *79*, 181-213.

Vinereanu, D. (2006). Risk factors for atherosclerotic disease: present and future. Herz *31 Suppl 3*, 5-24.

Voraberger, G., Schafer, R., and Stratowa, C. (1991). Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5'-regulatory region. Induction by cytokines and phorbol ester. J Immunol *147*, 2777-2786.

Wadgaonkar, R., Pierce, J. W., Somnay, K., Damico, R. L., Crow, M. T., Collins, T., and Garcia, J. G. (2004). Regulation of c-Jun N-terminal kinase and p38 kinase pathways in endothelial cells. Am J Respir Cell Mol Biol *31*, 423-431.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. Cell Death Differ *10*, 45-65.

Wang, G., Mao, J. M., Wang, X., and Zhang, F. C. (2004). Effect of homocysteine on plaque formation and oxidative stress in patients with acute coronary syndromes. Chin Med J (Engl) *117*, 1650-1654.

Wang, H., Jiang, X., Yang, F., Chapman, G. B., Durante, W., Sibinga, N. E. S., and Schafer, A. I. (2002). Cyclin A transcriptional suppression is the major mechanism mediating homocysteine-induced endothelial cell growth inhibition. Blood *99*, 939-945.

Wang, H., Yoshizumi, M., Lai, K., Tsai, J. C., Perrella, M. A., Haber, E., and Lee, M. E. (1997). Inhibition of growth and p21ras methylation in vascular endothelial cells by homocysteine but not cysteine. J Biol Chem *272*, 25380-25385.

Wang, Q., and Doerschuk, C. M. (2001). The p38 mitogen-activated protein kinase mediates cytoskeletal remodeling in pulmonary microvascular endothelial cells upon intracellular adhesion molecule-1 ligation. J Immunol *166*, 6877-6884.

Weibel, E. R., and Palade, G. E. (1964). New Cytoplasmic Components in Arterial Endothelia. J Cell Biol *23*, 101-112.

Weiss, N., Heydrick, S., Zhang, Y. Y., Bierl, C., Cap, A., and Loscalzo, J. (2002). Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathionine beta-synthase-deficient mice. Arterioscler Thromb Vasc Biol *22*, 34-41.

Weiss, N., Heydrick, S. J., Postea, O., Keller, C., Keaney, J. F., Jr., and Loscalzo, J. (2003). Influence of hyperhomocysteinemia on the cellular redox state--impact on homocysteine-induced endothelial dysfunction. Clin Chem Lab Med *41*, 1455-1461.

Weller, A., Isenmann, S., and Vestweber, D. (1992). Cloning of the mouse endothelial selectins. Expression of both E- and P-selectin is inducible by tumor necrosis factor alpha. J Biol Chem *267*, 15176-15183.

Werstuck, G. H., Lentz, S. R., Dayal, S., Hossain, G. S., Sood, S. K., Shi, Y. Y., Zhou, J., Maeda, N., Krisans, S. K., Malinow, M. R., and Austin, R. C. (2001). Homocysteine-

induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. J Clin Invest *107*, 1263-1273.

Whelan, J., Ghersa, P., Hooft van Huijsduijnen, R., Gray, J., Chandra, G., Talabot, F., and DeLamarter, J. F. (1991). An NF kappa B-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Nucleic Acids Res *19*, 2645-2653.

Wilcken, D. E., Wang, X. L., Adachi, T., Hara, H., Duarte, N., Green, K., and Wilcken, B. (2000). Relationship between homocysteine and superoxide dismutase in homocystinuria: possible relevance to cardiovascular risk. Arterioscler Thromb Vasc Biol *20*, 1199-1202.

Wilcken, D. E., and Wilcken, B. (1976). The pathogenesis of coronary artery disease. A possible role for methionine metabolism. J Clin Invest *57*, 1079-1082.

Wolff, B., Burns, A. R., Middleton, J., and Rot, A. (1998). Endothelial cell "memory" of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies. J Exp Med *188*, 1757-1762.

Woolhouse, I. S., Bayley, D. L., Lalor, P., Adams, D. H., and Stockley, R. A. (2005). Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease. Eur Respir J *25*, 612-617.

Wu, G. D., Lai, E. J., Huang, N., and Wen, X. (1997). Oct-1 and CCAAT/Enhancer-binding Protein (C/EBP) Bind to Overlapping Elements within the Interleukin-8 Promoter. THE ROLE OF Oct-1 AS A TRANSCRIPTIONAL REPRESSOR. J Biol Chem *272*, 2396-2403.

Wyble, C. W., Hynes, K. L., Kuchibhotla, J., Marcus, B. C., Hallahan, D., and Gewertz, B. L. (1997). TNF-[alpha] and IL-1 Upregulate Membrane-Bound and Soluble E-Selectin through a Common Pathway. Journal of Surgical Research *73*, 107-112.

Xia, Y., Tsai, A. L., Berka, V., and Zweier, J. L. (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. J Biol Chem *273*, 25804-25808.

Xia, Y., and Zweier, J. L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A *94*, 6954-6958.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science *270*, 1326-1331.

Xia, Z., Liu, M., Wu, Y., Sharma, V., Luo, T., Ouyang, J., and McNeill, J. H. (2006). N-acetylcysteine attenuates TNF-alpha-induced human vascular endothelial cell apoptosis and restores eNOS expression. Eur J Pharmacol *550*, 134-142.

Xu, D., Neville, R., and Finkel, T. (2000). Homocysteine accelerates endothelial cell senescence. FEBS Lett *470*, 20-24.

Xu, X., Heidenreich, O., Kitajima, I., McGuire, K., Li, Q., Su, B., and Nerenberg, M. (1996). Constitutively activated JNK is associated with HTLV-1 mediated tumorigenesis. Oncogene *13*, 135-142.

Yamada, K. M., and Geiger, B. (1997). Molecular interactions in cell adhesion complexes. Curr Opin Cell Biol *9*, 76-85.

Yamashita, K., Tasaki, H., Nagai, Y., Suzuka, H., Nihei, S., Kobayashi, K., Horiuchi, M., Nakashima, Y., and Adachi, T. (2005). Experimental hyperhomocysteinemia impairs coronary flow velocity reserve. Int J Cardiol *104*, 163-169.

Yi, F., Chen, Q. Z., Jin, S., and Li, P. L. (2007). Mechanism of homocysteine-induced Rac1/NADPH oxidase activation in mesangial cells: role of guanine nucleotide exchange factor Vav2. Cell Physiol Biochem *20*, 909-918.

Yi, F., Zhang, A. Y., Janscha, J. L., Li, P. L., and Zou, A. P. (2004). Homocysteine activates NADH/NADPH oxidase through ceramide-stimulated Rac GTPase activity in rat mesangial cells. Kidney Int *66*, 1977-1987.

Yuan, Q., Jiang, D. J., Chen, Q. Q., Wang, S., Xin, H. Y., Deng, H. W., and Li, Y. J. (2007). Role of asymmetric dimethylarginine in homocysteine-induced apoptosis of vascular smooth muscle cells. Biochem Biophys Res Commun *356*, 880-885.

Zalba, G., San Jose, G., Moreno, M. U., Fortuno, M. A., Fortuno, A., Beaumont, F. J., and Diez, J. (2001). Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension *38*, 1395-1399.

Zampolli, A., Basta, G., Lazzerini, G., Feelisch, M., and De Caterina, R. (2000). Inhibition of endothelial cell activation by nitric oxide donors. J Pharmacol Exp Ther *295*, 818-823.

Zeng, X., Dai, J., Remick, D. G., and Wang, X. (2003). Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes. Circ Res *93*, 311-320.

Zeng, X. K., Guan, Y. F., Remick, D. G., and Wang, X. (2005). Signal pathways underlying homocysteine-induced production of MCP-1 and IL-8 in cultured human whole blood. Acta Pharmacol Sin *26*, 85-91.

Zernecke, A., Bot, I., Djalali-Talab, Y., Shagdarsuren, E., Bidzhekov, K., Meiler, S., Krohn, R., Schober, A., Sperandio, M., Soehnlein, O., *et al.* (2008). Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. Circ Res *102*, 209-217.

Zhang, C., Cai, Y., Adachi, M. T., Oshiro, S., Aso, T., Kaufman, R. J., and Kitajima, S. (2001a). Homocysteine induces programmed cell death in human vascular endothelial cells through activation of the unfolded protein response. J Biol Chem *276*, 35867-35874.

Zhang, C., Kawauchi, J., Adachi, M. T., Hashimoto, Y., Oshiro, S., Aso, T., and Kitajima, S. (2001b). Activation of JNK and transcriptional repressor ATF3/LRF1 through the IRE1/TRAF2 pathway is implicated in human vascular endothelial cell death by homocysteine. Biochem Biophys Res Commun *289*, 718-724.

Zhang, D. X., and Gutterman, D. D. (2007). Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. Am J Physiol Heart Circ Physiol *292*, H2023-2031.

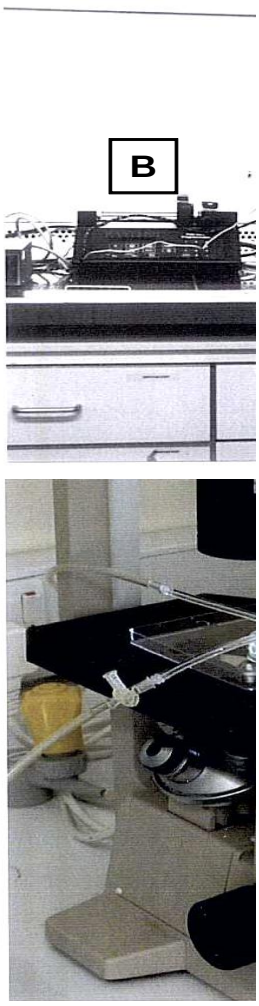
Zhang, H., Park, Y., Wu, J., Chen, X., Lee, S., Yang, J., Dellsperger, K. C., and Zhang, C. (2009). Role of TNF-alpha in vascular dysfunction. Clin Sci (Lond) *116*, 219-230.

Zhang, X., Li, H., Jin, H., Ebin, Z., Brodsky, S., and Goligorsky, M. S. (2000). Effects of homocysteine on endothelial nitric oxide production. Am J Physiol Renal Physiol *279*, F671-678.

Zhou, Z., Connell, M. C., and MacEwan, D. J. (2007). TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. Cell Signal *19*, 1238-1248.

APPENDIX 1

The *in vitro* system used to study HUVEC:neutrophil interactions under flow conditions.



The system is ready for flow experiment. A: camera attached on the top of the microscope. B: syringe pump through which neutrophils suspension is pulled. C: flow chamber is located on the microscope stage connected to the vacuum system (to the back) and to syringe installed on the syringe pump (to the right) and to the tube containing neutrophils (to the left).

APPENDIX 2

The buffers which have been used in western blot

Buffer 1 (Resolving gel): pH = 8.4

Reagents	**Grams/L**	**Grams/500ml**
1.5M Tris Base	181.5	90.75
0.4% w/v SDS	4	2

Buffer 2 (Stacking gel): pH = 6.8

Reagents	**Grams/L**	**Grams/500ml**
0.5M Tris Base	60.5	30.25
0.4% w/v SDS	4	2

Resolving Gel (amounts per gel):

		10%
H_2O	ml	9.6
Buffer 1	ml	6
Acrylamide	ml	8
TEMED N,N,N,N tetramethylene persulfate	µl	8
APS Ammonium persulfat	µl	90

Stacking Gel (amounts per gel):

		10%
H_2O	ml	9.75
Buffer 2	ml	3.75
Acrylamide	ml	1.5
TEMED	µl	20
APS	µl	150

Running Buffer

		1 Litre	**2 L**	**3 L**	**5 L**
Tris	G	3	6	9	15
Glycine	G	14.4	28.8	43.2	72
SDS (for final conc of 0.1%)	G	1	2	3	5

Blotting Buffer

		1 Litre	**2 L**	**3 L**	**5 L**
Tris	G	3	6	9	15
Glycine	G	14.4	28.8	43.2	72
Methanol	ml	200	400	600	1000

TBS-T (Tris Base Salline-T) pH = 7.4

		1 L	**2 L**	**3L**	**5L**
NaCl	G	8.78	17.56	26.3	15
Tris base	G	2.42	4.84	7.2	72
Tween	µl	1000	2000	3000	5000

ECL1

Luminol stock 1 ml
P-coumaric 0.44 ml
Tris base (1M, pH= 8.5) 10 ml

Continue to 100 ml with water

ECL2

H_2O_2 (30%) 64 µl
Tris base (1M, pH= 8.5) 10 ml

Continue to 100 ml with water

APPENDIX 3

Assessment of Hcy interaction with NAC using mass spectrometry

The following solutions were prepared and incubated overnight at 37°C and 5% CO_2 in humidified incubator

DL-Hcy (1mM)
NAC (1mM, pH=2.2) which is termed unbuffered NAC
NAC (1mM, pH=7.7 adjusted with 1M sodium bicarbonate) which is termed buffered NAC
Mix of DL-Hcy (1mM) and buffered NAC (1mM)
Mix of DL-Hcy (1mM) and unbuffered NAC (1mM)

Table 1: The different solutions prepared for Mass Spectrometry experiments

All stock solutions were prepared in dH_2O and then diluted (1:10) in 100% methanol before being infused into MS to detect any changes to the structure of these compounds once mixed after the indicated period of time. A negative control of 100% methanol was used to eliminate any non-specific background.

Mass spectrometry to detect interactions between Hcy and NAC

Mass spectrometry (MS) is an analytical technique used to determine the molecular mass of the sample and can give structural information of a sample or a molecule.

Micromass Quattro Ultima (triple quad mass spectrometry, Micromass UK Ltd, Manchester, UK) was used in this chapter to study any possibility for interactions between DL-Hcy and NAC which might lead to generating a third compound with different structure and properties from Hcy and NAC. Samples of Hcy and NAC at a concentration of 0.1mM (prepared as indicated above) were directly infused via a Harvard syringe pump at 10µl/min into the ionization source and then the components of the sample were ionized in the negative mode. Subsequently, ions will be directed to an electric field where the motion of the ions as they move within the electromagnetic field is translated into mass to charge ratio and thereafter, the molecular ion of each compound can be identified.

Settings of the machine were adjusted as follows: capillary set at 3.5 kilovolt, cone (35v) and desolvation temperature 150°C. For product scan of newly formed compounds (daughter fragmentation), a collision voltage of 5 was used. Molecule ionization was done in negative mode and therefore, the molecular weights of the detected compounds were calculated as follows:

MW = number displayed on the peak +1

Preliminary Results and Discussion

Mass spectrometry experiments were used to study any possibility that interactions between DL-Hcy and NAC which might reverse Hcy effects by altering its structure or by forming third compounds such as Hcy-NAC conjugates which might have less harmful effects on EC. The structure and mass to charge (m/z) values for Hcy and NAC and any expected new molecules which might have been formed in these experiments are displayed in Figure 53.

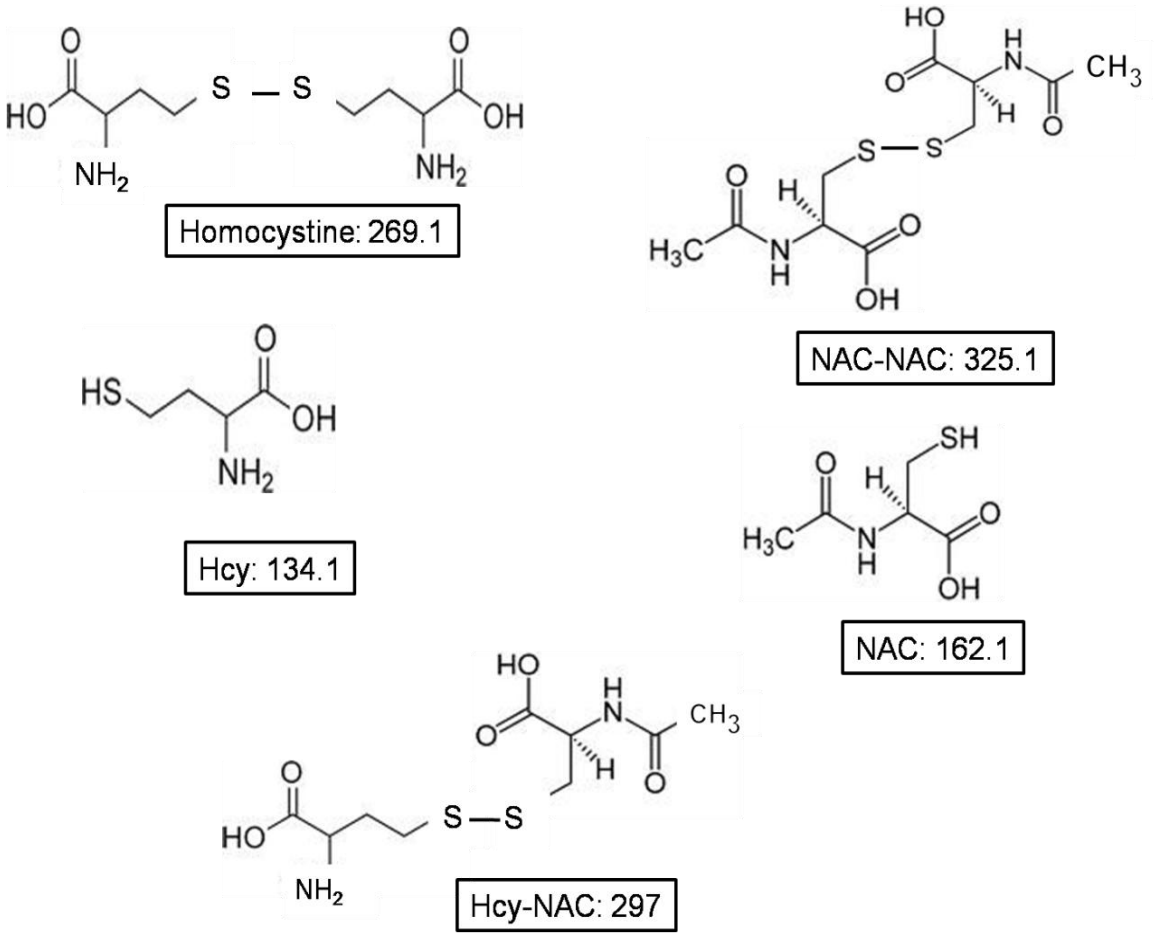


Figure 53: Chemical structures and m/z values of the molecules detected in ESI-MS (negative mode): Hcy, homocysteine (Hcy dimer), NAC, NAC dimer (NAC-NAC) and the expected structure for the 5th compound (Hcy-NAC).

As methanol was used to dilute all samples, 100% methanol alone was analyzed to eliminate any possible non-specific peaks and it showed only two peak (m/z 212 and m/z 127) (Figure 54). These are a background peaks possibly due to plasticizers in system. However, the m/z 127 ion did not appear in any of the other spectra which indicate that these data are unreliable. DL-Hcy solution showed three different significant peaks but one of them seems same as those of methanol. The smallest one represents Hcy while the

biggest one represents the dimer (homocystine). Hcy therefore is forming disulphide bonds with another Hcy through their free thiol groups to form homocystine molecule (Figure 55A). Unbuffered NAC solution also showed both monomer and dimer peaks (Figure 55B). Furthermore, the mix solution of (DL-Hcy and unbuffered NAC) showed both monomers and dimers of Hcy and NAC in addition to a third form compound (peak number of 297) (Figure 55C and Figure 56). The m/z value of the third ion suggests that it is formed from one molecule of Hcy and one molecule of NAC through a disulphide bond (Hcy-NAC). On the other hand, adjusting the pH of NAC solution with $NaHCO_3$ resulted in the disappearance of the NAC dimer in the buffered solution showing only the monomer to be present (Figure 57A). Furthermore, the solution of DL-Hcy and buffered NAC lacked the third compound (Hcy-NAC) (Figure 57C).

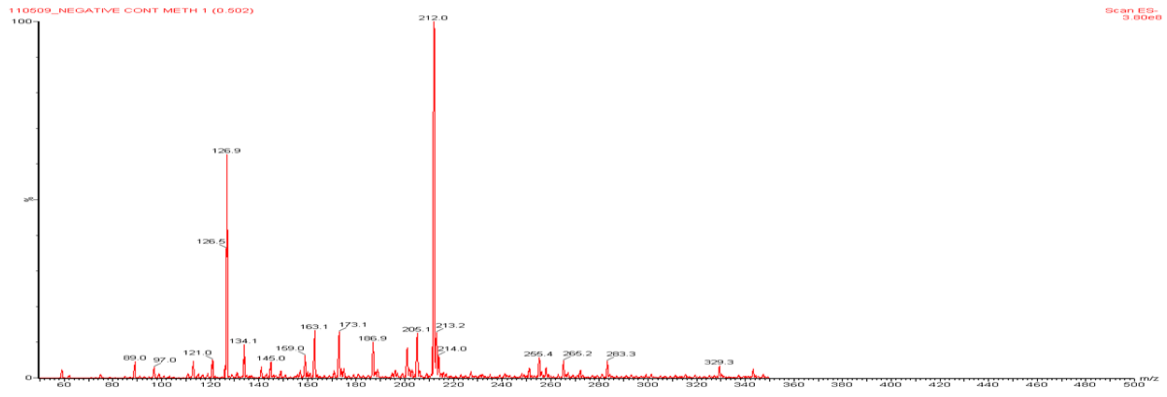


Figure 54: ES1-MS spectrum of the negative control (100% methanol).

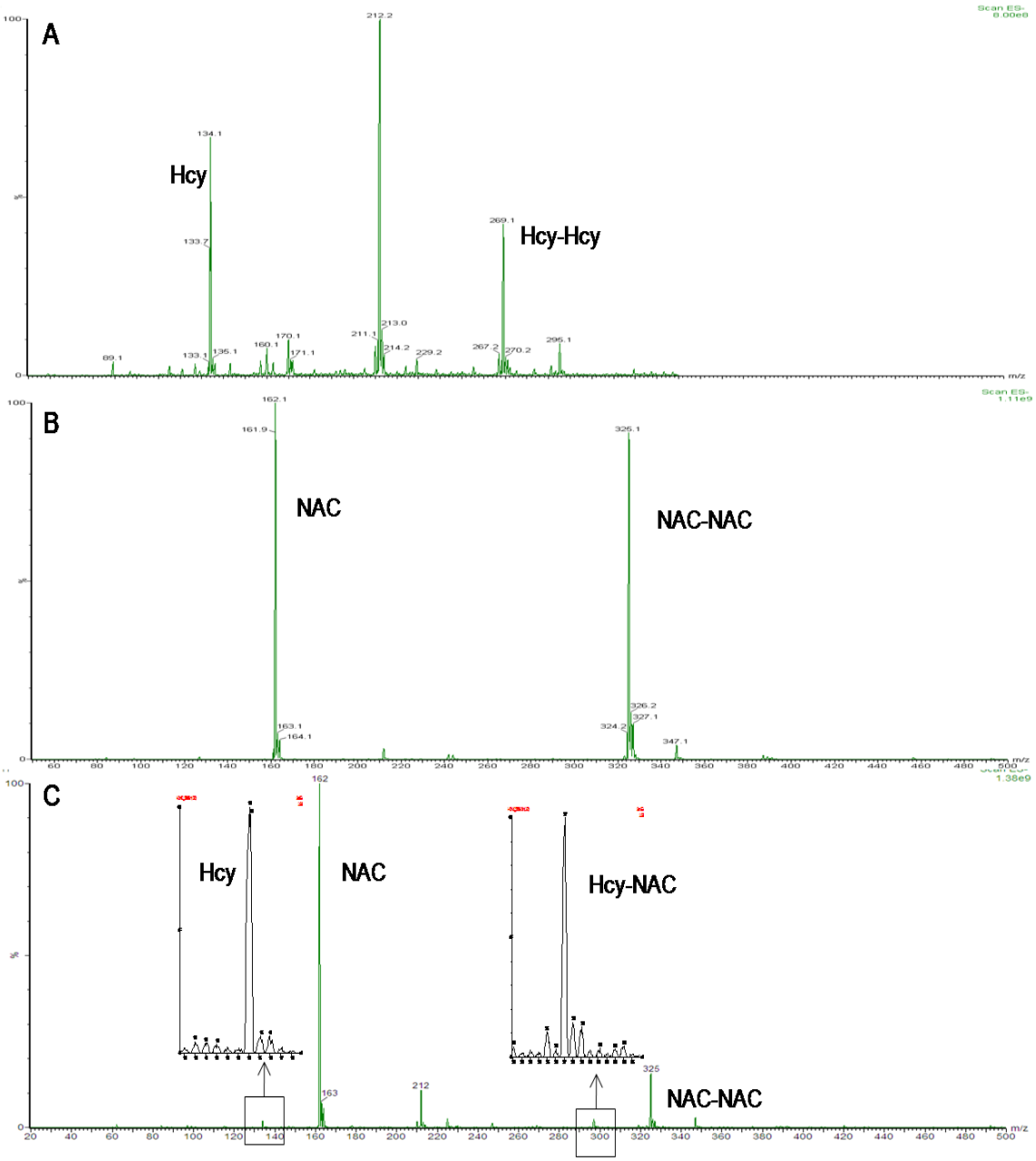


Figure 55: ES1-MS spectrum of: A: DL-Hcy alone, B: unbuffered NAC, C: DL-Hcy and unbuffered NAC.

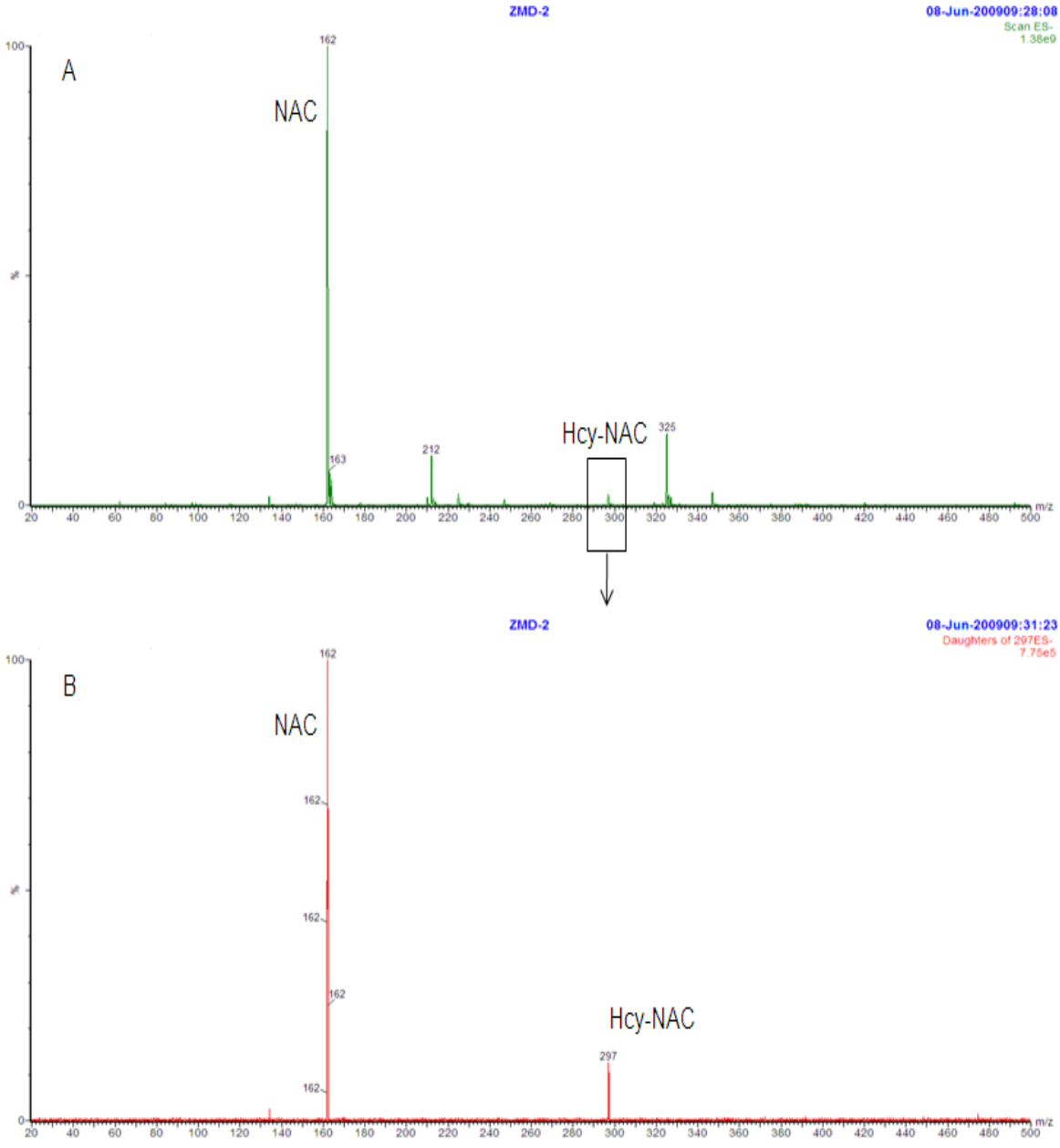


Figure 56: ES1-MS spectrum of: A DL-Hcy and unbuffered NAC. B: ES1-MS/MS spectrum of the third compound w/z 297.

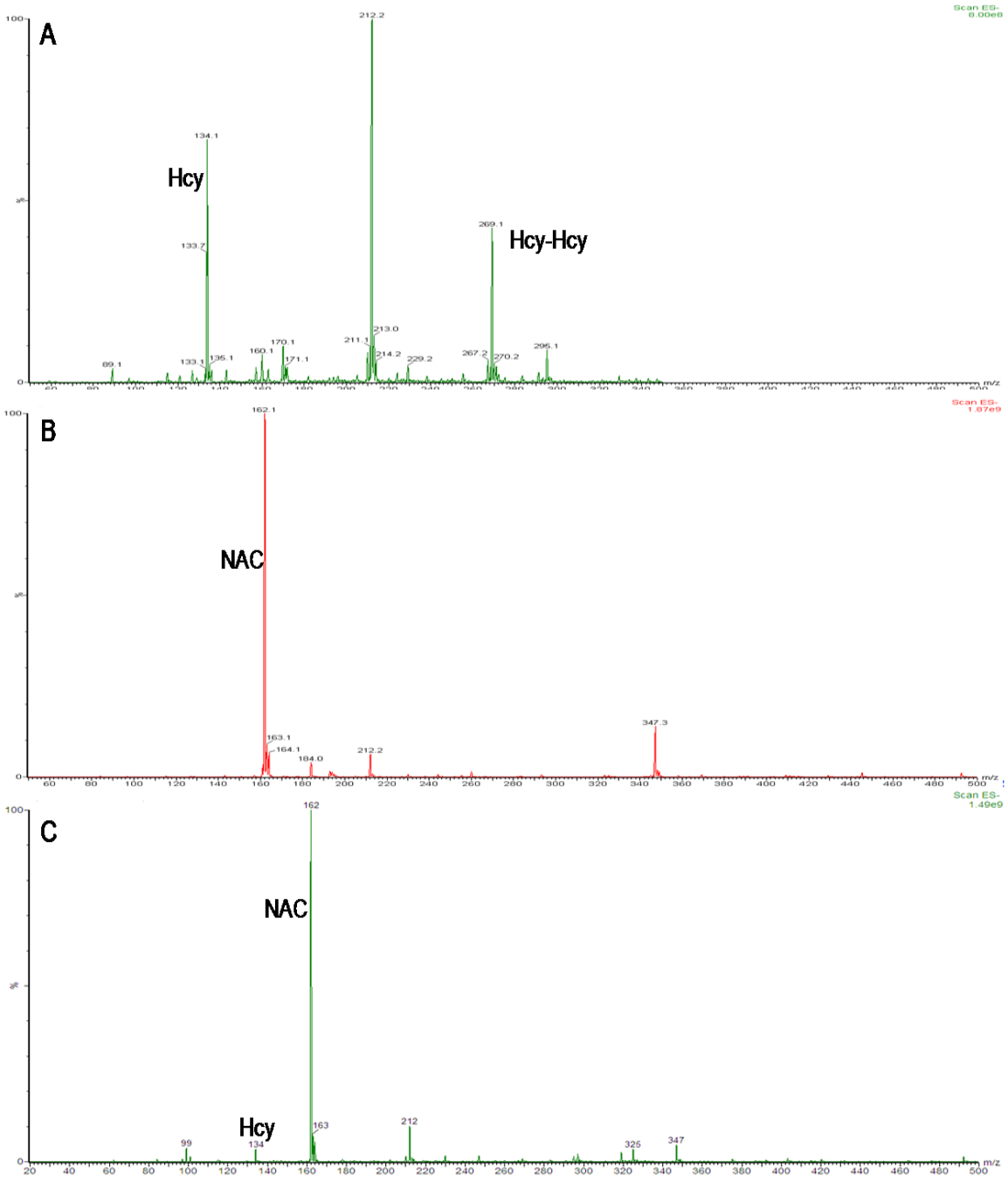


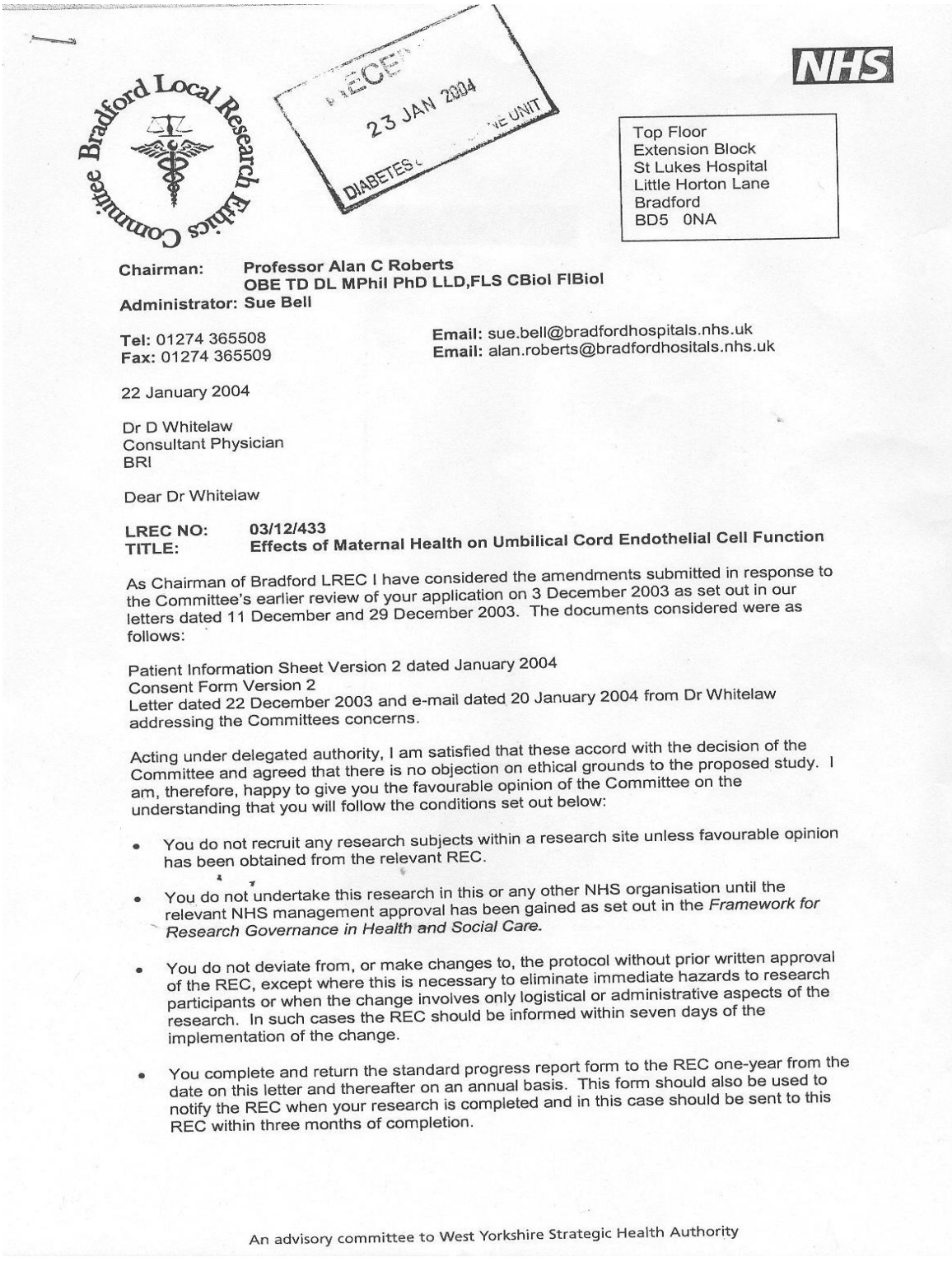
Figure 57: ES1-MS spectrum of: A: DL-Hcy alone, B: buffered NAC, C: DL-Hcy and buffered NAC.

We proposed that the thiol group of Hcy might form a disulphide bond with the thiol group of NAC forming a third compound (Figure 53) which might not be able to be taken up intracellularly, therefore reducing the intracellular uptake of Hcy. Decreasing Hcy cellular uptake would decrease its deleterious effects and that might present another mechanism by which NAC is preventing Hcy detrimental effects. To examine this theory, mass spectrometry was used to analyze the different compounds which are present in differently prepared solutions as explained in (Table 1). It would have been more relevant to study the interaction between Hcy and NAC in culture media supernatant. However, culture media supernatant contains many different compounds which can result in matrix effects and signal suppression and would make it very difficult to judge the possible formation of a new intermediate compound (Hcy-NAC). Therefore, the compounds were prepared in dH_2O and incubated in conditions similar to cell incubation conditions (37°C and 5% CO2 in humidified atmosphere). Furthermore, $NaHCO_3$ was used to buffer the NAC solution before adding it to cells in culture (pH 7.7). Therefore, and to study the effects of $NaHCO_3$ on the formation of the intermediate compound (Hcy-NAC), the solutions were prepared with buffered and unbuffered NAC (as explained in Table 1). Both NAC and Hcy were shown to be present in two different forms, monomer and dimer. However, the NAC dimer form was unstable at pH 7.7 (Figure 57). Furthermore, a third compound Hcy-NAC was detected in the unbuffered mix (Figure 55C and Figure 56). However, this third compound also disappeared once the pH was adjusted to 7.7 (Figure 57C). Therefore, it could be concluded that the dimers of Hcy and NAC were not stable at pH 7.7. Collectively, this preliminary data has shown that a third compound could be formed of Hcy and NAC together, however the stability of this compound is extremely

weak at physiological pH. This suggests that in culture media which has a pH of 7.7, the third compound is not likely to be formed and therefore Hcy structure won't be affected by the presence of NAC. Therefore, all the observed effects of NAC in reversing Hcy-induced JNK activation and also reversing Hcy-induced EC:neutrophil interactions most likely to be due to its effects as an antioxidant.

On the other hand, there are some limitations for the protocol designed to do these experiments. The contamination noticed in negative control (methanol) indicated that this control was unreliable and therefore invalidated the particular data. Furthermore, there might be some possible issues with the buffer used to prepare the solutions ($NaHCO_3$) as this buffer is not normally used in MS and would most likely induce matrix effects causing ion suppression. Alternately, ammonium bicarbonate could have been used because ammonium bicarbonate is compatible with mass spectrometry and its buffer range is (6.8-11.3). However, due to the fact that in experiments conducted on cells in culture, $NaHCO_3$ was used to buffer NAC solutions and thus, using another compound to buffer solutions in MS experiments will make the results incomparable. Therefore and as a future step, designing the experiments using the ammonium bicarbonate to buffer solutions in MS could be more relevant. Furthermore, in order to compare the *in vitro* results with the MS results, some of the *in vitro* experiments could be repeated, using ammonium bicarbonate, to check whether the results will be reproducible with different buffering solution.

APPENDIX 4



Bradford Local Research Ethics Committee

RECEIVED 23 JAN 2004 DIABETES UNIT

NHS

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St Lukes Hospital
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Bradford
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22 January 2004

Dr D Whitelaw
Consultant Physician
BRI

Dear Dr Whitelaw

LREC NO: **03/12/433**
TITLE: **Effects of Maternal Health on Umbilical Cord Endothelial Cell Function**

As Chairman of Bradford LREC I have considered the amendments submitted in response to the Committee's earlier review of your application on 3 December 2003 as set out in our letters dated 11 December and 29 December 2003. The documents considered were as follows:

Patient Information Sheet Version 2 dated January 2004
Consent Form Version 2
Letter dated 22 December 2003 and e-mail dated 20 January 2004 from Dr Whitelaw addressing the Committees concerns.

Acting under delegated authority, I am satisfied that these accord with the decision of the Committee and agreed that there is no objection on ethical grounds to the proposed study. I am, therefore, happy to give you the favourable opinion of the Committee on the understanding that you will follow the conditions set out below:

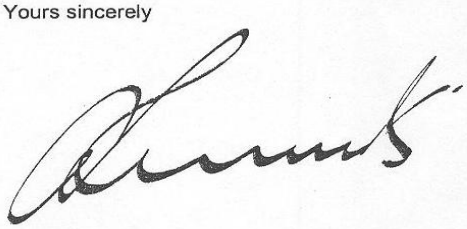
- You do not recruit any research subjects within a research site unless favourable opinion has been obtained from the relevant REC.
- You do not undertake this research in this or any other NHS organisation until the relevant NHS management approval has been gained as set out in the *Framework for Research Governance in Health and Social Care.*
- You do not deviate from, or make changes to, the protocol without prior written approval of the REC, except where this is necessary to eliminate immediate hazards to research participants or when the change involves only logistical or administrative aspects of the research. In such cases the REC should be informed within seven days of the implementation of the change.
- You complete and return the standard progress report form to the REC one-year from the date on this letter and thereafter on an annual basis. This form should also be used to notify the REC when your research is completed and in this case should be sent to this REC within three months of completion.

An advisory committee to West Yorkshire Strategic Health Authority

- If you decide to terminate this research prematurely you send a report to this REC within 15 days, indicating the reason for the early termination.
- You advise the REC of any unusual or unexpected results that raise questions about the safety of the research.

The project must be started within three years of the date on which REC approval is given.

Yours sincerely



Professor A Roberts
Chairman – Bradford Local Research Ethics Committee

Enc: Progress Report Form

APPENDIX 5

CHARACTARISTICS OF UMBILICAL ENDOTHELIAL CELLS FROM DIABETIC AND NON-DIABETIC MOTHERS

MOTHERS QUESTIONAIRE FORM

FOR CLINICAL USE ONLY
SAMPLE DATE: HOSPITAL No: CODE No

CONSENT Y N INFORMATION SHEET Y N

Age: Height Weight:

Ethnic group

Smoker: Y N No of years: past smoker: Y N

Prescribed drug use:

Recreational drug use: Y N please list

Diabetic: Y N No of years type:

Diabetic complications:

Medical conditions
during pregnancy

Other medical conditions
(e.g. asthma, heart disease):