

**MECHANISMS OF VASCULAR
PROTECTION IN CHRONIC
INFLAMMATORY DISEASE**

Thesis submitted for the degree of Doctor of Philosophy

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For Hector and Costas,
with love and gratitude

DECLARATIONS

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ABSTRACT

Premature cardiovascular disease (CVD) complicates chronic systemic inflammatory disease, and is initiated by endothelial dysfunction. Despite an ever-improving array of medications to treat these diseases, how to prevent CVD is not known. Studying the actions of endogenous mediators of endothelial cytoprotection and of disease-modifying drugs might establish how best to protect patients with systemic inflammatory diseases from atherosclerosis, if specific beneficial effects on vascular endothelium could be demonstrated.

High flow laminar shear stress (LSS) and vascular endothelial growth factor (VEGF) are two well known endogenous drivers of endothelial cytoprotection. Both can induce expression of protective genes but it is not known how confluent endothelial cells (EC) respond to VEGF when conditioned with LSS. Methotrexate (MTX) is the most widely prescribed treatment for rheumatoid arthritis and clinical data suggests that it reduces CV mortality. Known mechanisms of action of MTX result in intracellular accumulation of activators of AMP-activated protein kinase (AMPK). AMPK regulates cytoprotective genes in EC and its activation is associated with diverse desirable effects.

I hypothesised that EC conditioned with LSS would be primed to respond to VEGF, resulting in a synergistic induction of cytoprotective genes. Secondly I investigated whether MTX exerts beneficial protective effects on vascular endothelium via activation of AMPK, which enhance endothelial resistance to injury.

Studies of human umbilical vein EC (HUVEC) exposed to LSS showed that responses to VEGF are not enhanced in these conditions. However, MTX phosphorylated AMPK α^{Thr172} and induced expression of several cytoprotective genes, notably manganese superoxide dismutase (MnSOD). The addition of folic acid did not alter this; and it was also preserved when HUVEC were pre-treated with TNF α to mimic dysfunctional endothelium. siRNA depletion of AMPK attenuated MTX-mediated MnSOD induction. MTX treatment led to AMPK-dependent phosphorylation of the transcription factor CREB $^{\text{Ser133}}$. siRNA depletion of CREB also reduced MnSOD induction by MTX, and chromatin immunoprecipitation demonstrated binding of CREB to the MnSOD promoter in MTX-treated samples. Moreover, MTX protected HUVEC against apoptosis induced by glucose deprivation, demonstrating the functional importance of this pathway. Finally, treatment of the murine (BXSB x NZW) F_1 SLE model of inflammatory vasculopathy with MTX improved the intramyocardial arterial vasculopathy and reduced end-organ damage, increased aortic MnSOD and phosphorylated AMPK α^{Thr172} , and reduced ICAM-1 expression.

I have shown that MTX activates an AMPK-CREB pathway in vascular endothelium leading to enhanced expression of cytoprotective genes and protection against apoptosis *in vitro* and inflammatory vascular injury *in vivo*. This novel mechanism may explain its observed benefits in reducing CVD in chronic systemic inflammation.

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ABBREVIATIONS

A1	Bcl-2-related protein A1
ACC	Acetyl-CoA carboxylase
ACE	Angiotensin-converting enzyme
ACPA	Anti-citrullinated peptide antibodies
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated kinase
Ang-1	Angiopoietin-1
Ang II	Angiotensin II
ANOVA	Analysis of variance
AP-1	Activating protein-1
APC	Allophycocyanin
ApoE	Apolipoprotein E
APS	Anti-phospholipid antibody syndrome
ARE	Antioxidant response element
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BAEC	Bovine aortic endothelial cells
Bcl-2	B-cell lymphoma-2 protein
BH4	Tetrahydrobiopterin
BOEC	Blood outgrowth endothelial cells
BSA	Bovine specific albumin
CaMK	Ca ²⁺ /calmodulin-dependent protein kinase
CaMKK β	Ca ²⁺ /calmodulin-dependent protein kinase kinase- β
CBP	CREB-binding protein
CBS	

CD	Cluster of differentiation
cDNA	Complementary DNA
C/EBP	CCAAT-enhancer binding protein
ChIP	Chromatin immunoprecipitation
CMH ₂ DCFDA	5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
COX	Cyclo-oxygenase
CREB	Cyclic-AMP response element binding protein
CREM	Cyclic-AMP response element modulator
cT	Cycle threshold
cTnI	Cardiac troponin I
Cu/ZnSOD	Copper-zinc superoxide dismutase (SOD1)
CVD	Cardiovascular disease
DAF	Decay-accelerating factor
ddH ₂ O	Double-distilled water
DHFR	Dihydrofolate reductase
DMARD	Disease-modifying anti-rheumatic agent
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EC	Endothelial cells
ECGF	Endothelial cell growth factor
ECL	Enhanced chemiluminescence
ECSOD	Extracellular superoxide dismutase (SOD3)
EDTA	Ethylene diamine tetraacetic acid
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyogram
eNOS	Endothelial nitric oxide synthase

EPC	Endothelial progenitor cell
ERK	Extracellular-regulated protein kinase
ESR	Erythrocyte sedimentation rate
EULAR	European league against rheumatism
FA	Folic acid
FACS	Fluorescence activated cell sorting
FADH	Flavin adenine dinucleotide
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FOXO3a	Forkhead box O3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastro-intestinal
GPA	
HAEC	Human aortic endothelial cells
HBSS	Hanks' balanced salt solution
HCQ	Hydroxychloroquine
HDL	High density lipoprotein
H/E	Hematoxylin and eosin
HIF-1 α	Hypoxia-inducible factor-1 α
HMEC	Human microvascular endothelial cells
HMG-CoA	Hydroxymethylglutaryl coenzyme-A
HO-1	Heme oxygenase-1
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IGF-1	Insulin-like growth factor-1
ITP	Immune thrombocytopenic purpura

IVC	Inferior vena cava
JNK	c-Jun N-terminal kinase
KID	Kinase-inducible domain
KLF-2	Kruppel-like factor-2
LDL	Low density lipoprotein
LFA-1	Lymphocyte-function associated antigen-1
LKB1	Liver kinase B1
L-NAME	N (G)-nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
LSS	Laminar shear stress
MAC	Membrane attack complex
MEF	Myocyte-enhancer factor
MEK	Dual specificity mitogen activated protein kinase kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MI	Myocardial infarct
MMF	Mycophenolate mofetil
MnSOD	Manganese superoxide dismutase (= SOD2)
MOPS	3-(N-morpholino)-propanesulphonic acid
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NAC	N-acetyl cysteine
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κB
Nox	NADH oxidase
NQO-1	NAD(P)H dehydrogenase, quinone 1
Nrf2	Nuclear factor erythroid -2-related factor
NSAID	Non-steroidal anti-inflammatory drug
NZB	New Zealand black mouse

NZW	New Zealand White mouse
OD	Optical/ pixel density
oxLDL	Oxidised low density lipoprotein
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PE-Cy5	Phycoerythrin-cyanine 5
PerCP	Peridinin chlorophyll protein
PGC-1 α	PPAR γ coactivator -1 α
PI	Propidium iodide
PIC	Protease inhibitor cocktail
PI-3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulphonyl fluoride
PP2	Protein phosphatase-2
PPAR	Peroxisome-proliferator activated receptor
PsA	Psoriatic arthritis
PSS	Physiological saline solution
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RFI	Relative fluorescence intensity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXR	Retinoid-X receptor

SDS	Sodium dodecyl sulphate
siRNA	Short interfering ribonucleic acid
Sirt1	Sirtuin1
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SOD2	Manganese superoxide dismutase
TBS-T	Tris-buffered saline - tween
TGFβ	Transforming growth factor-β
TH4	Tetrahydrofolate
TLR	Toll-like receptor
TNFα	Tumour necrosis factor-α
Tris	Tris (hydroxymethyl) aminomethane
Trx	Thioredoxin
TSC2	Tuberous sclerosis complex-2
UCP-2	Uncoupling protein-2
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR2	VEGF receptor 2
WBF1	F1 of cross between NZW female and BXSB male mice
WHO	World Health Organisation
XO	Xanthine oxidase
Yaa	Y-linked acceleration area
ZMP	AICA ribonucleotide

1 INTRODUCTION

1.1 Atherosclerosis

1.1.1 Epidemiology

Atherosclerosis is a chronic disease of arterial vessel walls, leading to complications including myocardial and cerebral infarcts and aortic aneurysms. It is the commonest cause of death worldwide, with ischaemic heart disease and stroke together responsible for 22% of deaths in the world in 2011[1]. In the UK, according to the British Heart Foundation, cardiovascular disease (CVD) is responsible for more than a quarter of all deaths in the country each year: approximately 161 000. Coronary artery disease affects around 2.3 million people in the UK, and 103 000 have a myocardial infarct (MI) each year. The risk factors that determine the prevalence of atherosclerotic diseases were first demonstrated in the Framingham heart study and include cigarette smoking, hypertension, hypercholesterolaemia and diabetes mellitus[2-4]. More recently, it was discovered that chronic inflammation is also an important risk factor. Rates of CVD in the general population correlate with markers of systemic inflammation such as C-reactive protein (CRP)[5, 6], and an increased incidence of CVD is seen in many chronic inflammatory conditions[7].

1.1.2 Pathogenesis

Atherosclerosis affects predominantly large and medium-sized elastic and muscular arteries. The disease is slowly progressive and develops over several decades, a fact which places limitations on what can be learnt from *in vivo* models of the illness. The initiating event is injury to the endothelium, the monolayer lining all blood vessels, leading to endothelial dysfunction (discussed in detail in Section 1.3)[8]. Features of endothelial dysfunction include expression of cell adhesion molecules and increased permeability. These changes promote the adhesion and migration of leukocytes, particularly monocyte/ macrophages and T-lymphocytes, and the accumulation of cholesterol in the form of low-density lipoprotein (LDL) in the subendothelial intimal space, resulting in the earliest atheroma lesion: the fatty streak. Over the course of several years, activated macrophages engulf the LDL, which has been modified by oxidation, via their scavenger receptor. If macrophage cholesterol metabolism and efflux pathways become overwhelmed, foam cells are formed.

A chronic inflammatory process is then established in the vascular wall, leading to a self-perpetuating cycle whereby leukocytes secrete pro-inflammatory cytokines and chemokines that induce further cell adhesion molecule expression and promote chronic endothelial dysfunction, leading to greater recruitment of leukocytes and lipid. Smooth muscle cells and fibroblasts are also induced to migrate into the lesion and form an extracellular matrix that becomes a fibrous cap to the lesion, thus producing an atherosclerotic plaque. This protrudes into the lumen, causing a stenosis. This may

progress to a critical point at which ischaemia of the downstream tissue occurs; or the plaque may rupture acutely resulting in thrombus formation, complete vessel occlusion and tissue infarction[9].

1.1.3 Aetiology

As described above, atherosclerosis begins with injury to the endothelium, and thus it is factors that influence on endothelial cell (EC) biology that determine whether atheroma will develop. Three significant factors are hyperlipidaemia, pattern of blood flow and a chronic inflammatory milieu[10], although there are a plethora of others, comprehensively reviewed elsewhere[9].

Older theories of the causes of atherosclerosis emphasised lipid deposition within the plaque as the hallmark of the condition. Hyperlipidaemia is a key pre-requisite for the formation of atheroma; and evidence for this comes not only from the Framingham heart study, but also from the well known *in vivo* models of the disease such as the low density lipoprotein receptor (LDLR)^{-/-} and apolipoprotein E (ApoE)^{-/-} mice, which largely rely on hyperlipidaemia to drive atherogenesis. However, hyperlipidaemia cannot be the sole factor: 50% of MI occur in patients with normal serum cholesterol. It does not provide an explanation for the initiation of atherosclerosis, nor why intra-luminal lipid accumulates preferentially at branch points.

Atheroma forms exclusively in arteries, and has a clear geographical distribution. It has a predilection for parts of the arterial tree exposed to disturbed flow, such as branchpoints (e.g. the carotid bifurcation) and tight curves (e.g. the aortic arch), while those areas exposed to pulsatile laminar flow are rarely affected[11]. This suggests that the blood flow patterns and the shear stress exerted on the vessel wall are critical for determining susceptibility to atherogenesis. As it is the endothelium that is exposed to and senses shear stress, it is thought to be the mediator of this phenomenon, which will be discussed in section 1.4. Thus, in the "response to injury" hypothesis of Ross, turbulent blood flow renders the endothelium susceptible to injury and less able to mount a protective response when exposed to harmful factors including LDL, hyperglycaemia, pro-inflammatory cytokines and hyperhomocysteinaemia[8]. Endothelial dysfunction, allowing the tracking of leucocytes and lipid into the subendothelial space, therefore predominantly occurs here.

That inflammation is important for the initiation of atheroma and for the perpetuation of lesions is supported by a multitude of differing lines of evidence. Firstly, atherosclerosis is more common in people with raised markers of systemic inflammation such as CRP[5]. Secondly, atherosclerosis is more common in patients with other chronic systemic inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[12]. Thirdly, a world-wide genetics consortium has shown that individuals with an interleukin (IL)-6 receptor polymorphism that reduces IL-6 signalling have a reduced incidence of coronary heart disease[13]. Finally, patients with raised CRP benefit more from aspirin in terms of cardiovascular risk reduction than those with normal values[5], while the JUPITER trial showed that rosuvastatin reduced CV events in patients with

normal serum cholesterol but raised CRP, measured by high-sensitivity assay[14]. These two findings suggest that risk reduction is being achieved through a reduction in inflammation rather than solely through anti-platelet effects or reductions in serum cholesterol, and that reducing inflammation could itself be a useful strategy to tackle atherosclerosis.

1.1.4 Current treatment paradigms

Treatment for atherosclerosis involves two major strategies. Firstly, disease progression can be reduced by modification of risk factors such as smoking, hypertension, dyslipidaemia, treatment of diabetes, diet and exercise. Secondly, atherosclerotic plaques that have enlarged until they cause symptomatic restriction of blood flow, or those that rupture acutely, are dealt with by intervention to reopen the blocked vessel, through percutaneous coronary intervention or thrombolysis, or by bypass graft surgery if medical intervention is unsuccessful or not feasible.

There is no treatment as yet specifically targetting the process of atherogenesis itself. However, given the strong evidence suggesting that atherosclerosis is a chronic inflammatory condition, two new trials have recently started recruiting patients. These are designed to test the hypothesis that atherosclerosis is a chronic inflammatory disease of large arteries, and will involve treating patients who have already suffered myocardial infarcts and who have raised CRP or the metabolic syndrome with methotrexate (MTX)[15] or canakinumab[16]. Canakinumab is a monoclonal antibody that inhibits IL-1 β , an archetypal pro-inflammatory cytokine. MTX is a well-established treatment for RA and other chronic inflammatory diseases, with a wide variety of anti-inflammatory actions, and will be discussed in detail later in the chapter. The aim of these trials is to show whether direct targetting of inflammation can prevent complications of atherosclerosis, thus proving that atherosclerosis is a disease perpetuated by inflammation.

1.2 Atherosclerosis in chronic systemic inflammation

As described earlier, atherosclerosis is an inflammatory condition, and it occurs more commonly in people with other chronic inflammatory diseases. This section discusses the increase in atherosclerosis associated with systemic inflammation, and the mechanisms underlying this in RA and SLE.

1.2.1 Rheumatoid arthritis

RA is a chronic systemic inflammatory condition characterised by symmetrical polyarticular synovitis that progresses to joint destruction. It affects approximately 0.8% of the general population in the UK[17]. Central to its pathogenesis is systemic and synovial synthesis of pro-inflammatory cytokines such as tumour necrosis factor (TNF α), IL-6 and IL-17, B and T-lymphocyte activation, and production of auto-antibodies including rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA)[18].

CVD is 50% more common in patients with RA than in age and sex-matched healthy controls and is the major cause of death in this illness[19]. In some studies the increased incidence is up to 4-fold, making RA equivalent to diabetes mellitus as a risk factor for MI and stroke[20, 21].

The mechanisms underlying the increased incidence of CVD in RA are heterogeneous. Firstly, the traditional Framingham risk factors for atherosclerosis are more common in patients with RA, although this does not explain the extent of the increased incidence[22]. Tobacco smoking is a risk factor for the development of ACPA and of seropositive RA[23, 24], but smoking status does not precisely correlate with CV events in RA and thus cannot fully explain the increase (REF). Insulin resistance and the metabolic syndrome are also more prevalent in RA[25, 26], and may be related to high circulating levels of pro-inflammatory cytokines[27]. Thirdly, RA is associated with a distinct pattern of dyslipidaemia, with low high-density lipoprotein (HDL) and LDL cholesterol and raised triglycerides[28, 29]. HDL in RA can be abnormal, with higher levels of oxidised pro-inflammatory HDL. This could promote atherogenesis through decreased reverse cholesterol transport[30] and decreased paraoxonase activity, an antioxidant enzyme present in HDL particles that normally prevents HDL and LDL oxidation[31].

In addition to the increased incidence of traditional risk factors for atherosclerosis, there are disease-specific mechanisms at work that promote atherosclerosis in RA. These include the effects of long-term pro-inflammatory cytokine exposure, autoantibodies and a T-lymphocyte subset specific to RA.

The principal pro-inflammatory cytokines that are thought to be involved in vascular injury in RA are TNF α , IL-6 and IL-17. Serum TNF α levels are increased in RA and correlate with coronary artery calcification[32]. TNF α is likely to be important in the initiation and progression of atheromatous lesions in the general population as well as in RA, as its actions include induction of endothelial cell adhesion molecules and chemotactic proteins that promote mononuclear cell invasion of the sub-

endothelial space, and macrophage scavenger receptor expression to promote foam cell formation[33]. RA serum can induce similar changes in macrophages and its effects can be partially reversed by anti-TNF α or IL-6 antibodies, emphasising their importance for endothelial injury in RA[33].

IL-6 is the most abundant cytokine in rheumatoid synovium and an important contributor to disease pathogenesis. In RA, increased IL-6 correlates with increased CVD, independently of traditional variables[32]. It is also found in human atheromatous plaques in non-RA patients[34] and exacerbates atherogenesis in ApoE^{-/-} mice[35].

IL-17 is pathogenic in RA synovial inflammation and may also be important in RA-associated CVD. Levels of IL-17 are an independent predictor of poor microvascular endothelial function and macrovascular arterial compliance in RA patients with stable disease[36]. IL-17 blockade protects against atherogenesis in LDLR^{-/-} mice[37]. Mechanistically, the combination of IL-17 and TNF α together on EC appears to have a synergistic pro-inflammatory effect and induces pro-thrombotic gene expression[38].

Autoantibody production is a central feature of RA. The presence and titre of RF has been associated with CV mortality in the general population, after controlling for systemic inflammatory response markers such as erythrocyte sedimentation rate (ESR)[39]. A correlation between RF and ACPA and impaired endothelial function and carotid intima-media thickness has been reported but not all studies agree[40-42]. It remains unclear whether rheumatoid auto-antibodies have a direct role in atherogenesis or are solely markers of disease severity and thus correlate with development of CVD.

Finally, the presence of a subset of T-cells (CD4⁺ve CD28⁻ve), which are associated with more aggressive disease in RA, has been reported to correlate with endothelial function and carotid intima-media thickness[43]. They have also been found in patients suffering unstable angina but not stable angina, possibly indicating a common pathology linking RA and vulnerable atherosclerotic plaques[44]. However, it is not known whether this T-cell population plays a direct role in atherosclerotic plaque development in RA patients.

1.2.2 Systemic lupus erythematosus

SLE is a multi-system autoimmune disorder with widely varying clinical features caused by immune-mediated damage to internal organs. It occurs predominantly in pre-menopausal women, a population generally thought to be protected against CVD. Despite this, an increased incidence of CVD is a prominent complication of the condition[45]. However, active SLE is not strongly associated with increased canonical pro-inflammatory markers such as CRP that correlate in both RA and the general population with CVD risk. This suggests that the mediators of increased CVD in SLE differ from those that operate in RA and the general population[12].

SLE in young women is associated with an up to 50 fold increased relative risk of CVD[46]. A bi-modal distribution of mortality is reported in the disease, with the later peak thought to be due predominantly to atherosclerosis[47]. As with RA, increased risk of CVD is seen with longer disease duration, supporting the idea that long term exposure to the immune abnormalities of SLE promote atherogenesis[48]. Abnormalities in endothelial function can appear early in the course of disease[49] and a substantial proportion have evidence of subclinical atherosclerosis[50].

There are diverse reasons for the increased incidence of CVD in SLE. Traditional Framingham risk factors are important but do not account fully for the extent of atherosclerosis[51]. Smoking appears to be the traditional risk factor with the biggest contribution[52]; but there is also a high incidence of hypertension in SLE due to nephritis, and lipid profiles in SLE are frequently abnormal, with low HDL, raised LDL and raised pro-inflammatory HDL. The latter is more prevalent in SLE than RA and correlates with subclinical atherosclerosis[30]. In addition, microalbuminuria, insulin resistance and hyperhomocysteinaemia may all contribute, being present with increased frequency in SLE than control populations[53].

Disease-specific factors promoting CVD in SLE include increased circulating interferon- α (IFN α) levels and autoantibody production. The classic pro-inflammatory cytokines such as IL-6 and TNF α are not so important in SLE pathogenesis as in RA, instead IFN α may play a role in driving SLE inflammation with increased levels seen in active disease[45]. Patients with increased IFN α -driven gene expression have more severe endothelial dysfunction and coronary artery calcification[54], and reducing Type I IFN levels in atherosclerosis-prone mice reduces plaque size[55]. This suggests that raised IFN α , as found in active SLE, promotes atherogenesis and that this plays a role in vascular injury.

Three mechanisms have been suggested by which increased circulating IFN α may contribute to CVD in SLE, shown in animal and human studies. Firstly, IFN α reduces the numbers and function of circulating endothelial progenitor cells (EPC), thus damaging intrinsic mechanisms of vascular repair[55], possibly by affecting the angiogenic factors vascular endothelial growth factor (VEGF) and IL-1 β [56]. Secondly, IFN α may have roles in plaque progression. IFN α signalling and plasmacytoid dendritic cells making IFN α are increased in atheromatous plaques[57]. IFN α can increase macrophage infiltration of vessel walls[58] and promotes foam cell formation by increasing oxidised LDL uptake and modification of scavenger receptors[59]. Finally, it can also promote thrombosis by increasing platelet activation and platelet-leukocyte aggregation, in murine models of SLE[55].

Several groups of autoantibodies may be pertinent to the development of atherosclerosis in SLE. Firstly, anti-phospholipid antibodies are associated with the development of arterial thrombosis and titres correlate with carotid intima-media thickness[60]. However, a firm causal link with CVD has

not yet been shown. Secondly, antibodies to HDL and to apolipoprotein A1 (present in HDL particles) are found in serum from SLE patients[61]. These may interfere with the role of HDL in reverse cholesterol transport, thus worsening plaque cholesterol deposition. Finally, titres of anti-Ro antibodies, one of the common antibodies to nuclear proteins found in SLE, correlate with EPC dysfunction, although it is not known if they have a direct pathogenic role[62].

1.2.3 Other chronic inflammatory disorders

In addition to RA and SLE, an increased risk of atherosclerosis has been demonstrated in other chronic inflammatory conditions: either an increased incidence of coronary artery disease or of subclinical atherosclerosis. The relative rarity of many of these conditions means that the large population studies needed to show conclusively that rates of CVD are increased are difficult to perform, hence, in many cases, markers of subclinical disease such as carotid intima-media thickness have been studied.

Both plaque psoriasis and psoriatic arthritis (PsA) increase the risk of ischaemic heart disease and coronary artery events in a recent meta-analysis[63] and systematic review[64]. However, the relative risk of CVD appears lower than that associated with RA, and neither study demonstrated an association with CV mortality.

In ankylosing spondylitis (AS), a large retrospective cohort study involving 8600 Canadian patients demonstrated a small increase in disease-associated ischaemic heart disease and cerebrovascular disease. The difference between AS patients and the general population was most pronounced in young patients[65]. A more recent prospective study has since confirmed that ischaemic heart disease is more prevalent in AS compared to the general population[66].

A recent meta-analysis examining CVD in inflammatory bowel disease, which included 33 observational studies containing more than 200 000 patients, showed an increased risk of ischaemic heart disease but not of CV mortality[67]. As with AS and PsA, the increased risk was small: a relative risk increase of 25-50%.

Systemic sclerosis has recently been shown in two retrospective cohort studies to be associated with an increased risk of acute myocardial infarction, stroke and peripheral vascular disease[68, 69]. An approximate doubling in the risk was observed in both.

Primary systemic vasculitis, particularly granulomatous polyangiitis (GPA), has been linked with premature atherosclerosis. Faurschou *et al* found a doubling in the expected rate of acute myocardial infarction among 200 patients with GPA[70].

Finally chronic infection, particularly periodontal disease, is associated with increased rates of coronary atherosclerosis and carotid intima-media thickness[71, 72]. This observation also extends to chronic infections in general[73].

Thus, it can be seen that chronic inflammation, of widely differing origins, is associated with the development of atherosclerosis. As with RA and SLE, the mechanisms that contribute may be specific to each condition, but all converge on a common pathway of endothelial damage, leading ultimately to atherogenesis.

1.2.4 Current strategies to reduce atherosclerosis in systemic inflammation

Prevention of atherosclerosis in systemic inflammation is at present suboptimal. This is because the drivers of vascular damage in each disease are not fully understood and so cannot be specifically treated. Secondly, it is not clear whether the measures adopted in other chronic diseases including type II diabetes, modification of the Framingham risk prediction tables, can be straightforwardly transferred. Finally, although treatments for SLE and RA are improving rapidly, it is not as yet clear whether effective treatment of the disease reduces CVD. With many of these conditions, the underlying immune abnormalities persist for many years longer than the overt disease phenotype[74], and so vascular damage could accrue even when there is outwardly no active inflammatory disease.

Current best clinical practice is to manage optimally the traditional risk factors for CVD. The European League Against Rheumatism (EULAR) recommendations in RA are to multiply the conventional risk prediction scores by 1.5 in patients with RA for more than 10 years or who are seropositive, so lowering the threshold for treatment; and to monitor modifiable risk factors such as dyslipidaemia on an annual basis[75]. Statins in RA reduce serum cholesterol and improve CV and all cause mortality in primary prevention[76]. In SLE the benefits of statins are less clear: they improve endothelial function in the short term, but at 2 year follow-up no improvement in coronary calcification score or carotid atherosclerosis was seen[77, 78].

Although management of RA in general has vastly improved over the last 20 years, there has as yet been no proof of a reduction in the rate of CV complications with improved disease management. However, specific medications used in the treatment of RA may be beneficial. There is good evidence that use of MTX is associated with a reduction in the rate of MI and of CV mortality[79]. Hydroxychloroquine (HCQ) improves lipid profile in RA and reduces carotid plaque in SLE [80, 81]. There is less conclusive evidence for other drugs, particularly leflunomide[82], probably because they are prescribed at lower frequency and thus research data on their effects in clinical practice is harder to obtain. The effects of anti-TNF α biologic agents on atherosclerosis have been studied but whether they lower risk or not remains unclear. A recent meta-analysis suggested a trend towards cardioprotection, but this was significant only in cohort studies and not in randomised controlled trials[83].

In SLE, effective disease treatment in general seems to reduce the progression of atherosclerosis. Lower cumulative doses of azathioprine, cyclophosphamide and prednisolone may be associated with more CVD in later life[48], and aortic atheroma is reduced in patients given cyclophosphamide

compared to those who have never received it[81]. Mycophenolate mofetil (MMF) may be beneficial: it reduces carotid plaque in patients with symptomatic carotid artery stenoses and reduces atherogenesis in LDLR^{-/-} mice crossed with lupus-prone mice[84, 85].

Certain other classes of drugs used to treat systemic inflammatory diseases may have harmful vascular effects. Non-steroidal anti-inflammatory drugs (NSAIDs) are thought to increase the risk of myocardial infarction in the general population, but so far have not been shown to do so in RA, suggesting that their anti-inflammatory effects predominate[86]. Glucocorticoid steroids have a long term adverse influence on glucose homeostasis and blood pressure that one would expect to worsen vascular risk, but are such good anti-inflammatory drugs that the overall effect on the vasculature remains unclear, despite decades of widespread use[12].

Although strategies do exist to limit CVD in inflammatory disease, these at present consist only of aggressively targetting the known modifiable risk factors. Although we have evidence that some immunosuppressive and disease-modifying drugs may reduce CVD, we have no good idea how and indeed whether this would be enough to lower risk to the background population level. There is therefore an outstanding need for a clinical strategy to protect the vasculature in chronic inflammatory disease, and given that atherogenesis begins with endothelial injury, understanding the mechanisms of endothelial injury and how these can be combatted would be a good place to start.

1.3 Endothelial dysfunction

1.3.1 Functions of normal endothelium

The vascular endothelium forms a continuous monolayer lining the entire vasculature from endocardium to capillary bed, may weigh up to 1.5kg and covers between 1 and 7m² in surface area[87]. It was originally thought simply to form a passive barrier between the bloodstream and the interstitium, allowing the diffusion of oxygen, nutrients and waste products. Research over the last few decades, however, has shown it to be a highly active tissue, central to the regulation of many physiological and pathological processes[88, 89]. The functions of the endothelium as they are now understood include:

- Control of vasomotor tone through the secretion of vasoconstrictor (e.g. endothelin-1 and angiotensin II) and vasodilative (e.g. nitric oxide (NO) and prostacyclin) factors;
- Maintenance of blood fluidity through roles in the coagulation cascade and fibrinolysis. The endothelium is normally coated in a glycocalyx mesh composed of heparan sulphate and hyaluronan which presents an anti-thrombotic surface to the bloodstream;
- Formation of a selective barrier between bloodstream and interstitial space, permeable to oxygen, nutrients, waste products and other molecules as needed;
- Angiogenesis, through the production of angiopoietin-1 (Ang-1), VEGF and platelet-derived growth factor (PDGF);
- A key role in innate and adaptive immunity through regulation of leucocyte trafficking by the inducible expression of cell adhesion molecules including E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)[87-89].

1.3.2 Clinical features of endothelial dysfunction

As discussed earlier, current theories of the aetiology of atherosclerosis postulate that the disease begins with endothelial injury, leading to endothelial dysfunction[8]. Endothelium is normally a quiescent tissue with low cell turnover, promoting vasodilatation through NO production and release, and resistant to the activation of inflammatory and thrombotic processes. Endothelial dysfunction denotes a change in phenotype to promote vascular inflammation, vasoconstriction and coagulation and its principal feature is a reduction in the bioavailability of NO, or an imbalance between NO and other factors that antagonise its actions[90]. It is also associated with increased reactive oxygen species (ROS), cell adhesion molecule expression and increased EC apoptosis[12, 91].

Endothelial dysfunction not only precedes atherosclerosis, it is also intimately involved in the progression of plaque formation[8]. Importantly from a clinical perspective, it is detectable in asymptomatic individuals without clinically evident atherosclerosis[92], independently predicts ischaemic events[93], and is reversible[94]. Identifying successful ways to combat endothelial

dysfunction therefore holds promise as a way to retard the development and progression of atherosclerosis.

The causes of endothelial dysfunction are essentially the same pathogenic processes that are known to be associated with the development of atherosclerosis, including hypertension, hyperlipidaemia, diabetes mellitus, tobacco consumption, obesity, and chronic systemic inflammation. Accordingly, mediators such as oxidised LDL, cigarette smoke, angiotensin II, hyperglycaemia and pro-inflammatory cytokines such as TNF α , IL-1 and IL-6 have all been demonstrated to have adverse effects on EC *in vitro* and endothelial responses *in vivo* that are consistent with promoting a dysfunctional endothelium[8, 91].

Individuals with endothelial dysfunction are essentially asymptomatic. Clinical detection is therefore difficult, and the techniques to do so are time consuming and relatively invasive so thus far have been confined to use as research tools. There are many different ways to assess endothelial function in patients, reviewed comprehensively by Khan *et al*[90]. These include non-invasive assessment of flow-mediated dilatation in the brachial artery; vasodilation of the coronary vessels to acetylcholine infusion during coronary angiography, and assessment of the microcirculation using Laser doppler flowmetry combined with iontophoresis of acetylcholine. In addition, analysis of plasma levels of endothelial proteins such as E-selectin, thrombomodulin and von Willebrand factor has been used.

Given the importance of endothelial dysfunction for the initiation and perpetuation of atherosclerosis, it is likely that an increased incidence of it contributes to the increased rate of CVD in chronic inflammation. Several studies have shown that endothelial dysfunction is seen in RA, in patients without other risk factors for CVD or clinically evident disease[95], and in patients with recent disease onset[96, 97] and with ostensibly well-controlled disease[98]. Endothelial dysfunction has also been noted in SLE[99], PsA[100] and primary systemic vasculitis[101] and can be reversed or improved by treatment of the disease, at least temporarily[102]. Its presence at diagnosis and in otherwise well-controlled disease underscores the difficulty in successfully combatting it simply by treating the illness itself.

1.3.3 Pathophysiology of endothelial dysfunction

While normal endothelium has an anti-coagulant, anti-inflammatory phenotype and exerts a tonic vasodilatory effect, endothelial dysfunction denotes a vascular condition that is characterised by vasoconstriction, inflammation and predisposes towards thrombosis. This section discusses the pathogenic processes that lead to this. The mechanisms underlying this change include reduced NO availability, expression of cell adhesion molecules, increased EC apoptosis and reduced mechanisms of EC repair (Figure 1.1). A common feature of many of these disordered processes is increased

oxidative stress, which will be addressed in the next section.

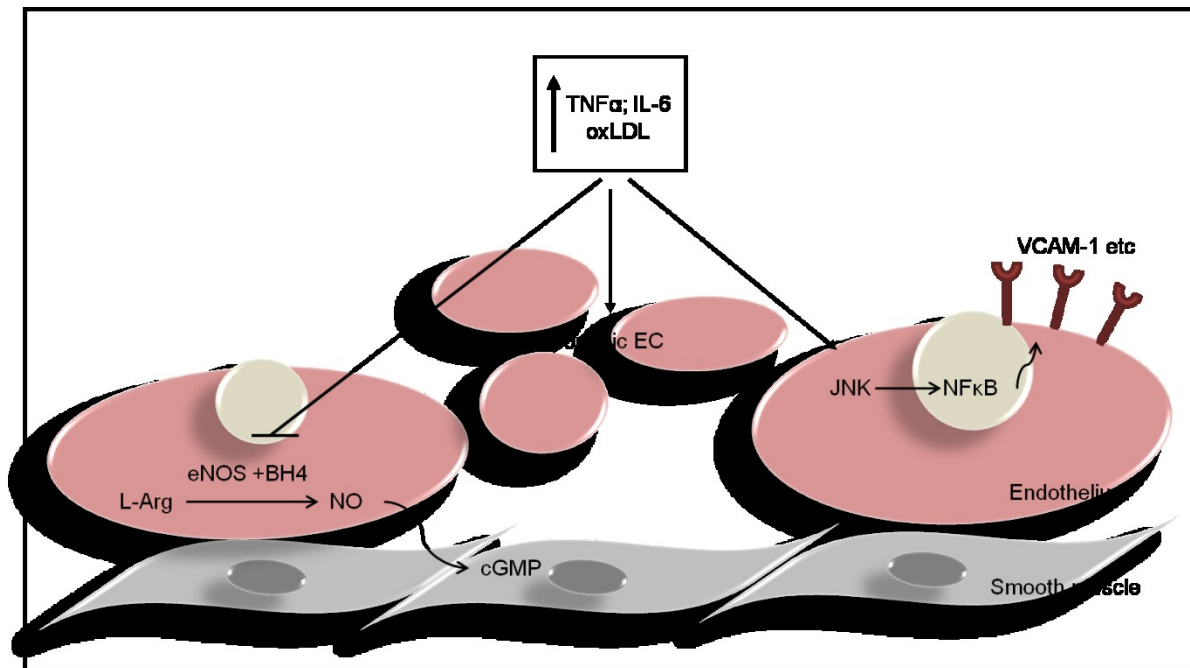


Figure 1.1 Pathophysiology of endothelial dysfunction.

Reduced NO bioavailability

As it is currently understood, the central pathological characteristic of endothelial dysfunction is reduced NO bioavailability. NO is synthesised and released by EC and is the most physiologically important vasodilatory factor. It also has multiple protective functions beyond its vasomotor role, which relate closely to the characteristics of normal vascular endothelium. Loss of NO therefore, in addition to predisposing to hypertension through vasoconstriction, leads to increased platelet adherence and thus thrombosis, smooth muscle proliferation and migration and thus hypertension, as well as atherosclerosis, leucocyte adhesion and oxidative stress[91].

NO is formed through metabolism of L-arginine into L-citrulline and NO, catalysed by endothelial nitric oxide synthase (eNOS)[87]. eNOS can be activated in two ways: firstly, by binding calmodulin, which occurs through rises in intracellular calcium, and secondly by phosphorylation on serine 1177. The reaction requires two co-factors: nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH4). Vasodilator hormones raise intracellular calcium and thus increase eNOS activity; fluid shear stress affects eNOS activity through protein kinase A (PKA) or Akt-dependent phosphorylation, although a number of other kinases, including AMP-activated kinase (AMPK), are also capable of eNOS phosphorylation on serine 1177[91].

The best known function of endothelial NO production is vasodilation. This occurs through diffusion of NO from endothelium to smooth muscle cells in the arterial media, where it activates guanylate cyclase, leading to a rise in cyclic guanosine monophosphate (cGMP). This mediates smooth muscle relaxation[87].

A reduction in NO bioavailability may occur through multiple mechanisms in chronic inflammatory conditions. Inflammation can cause shearing of the endothelial glycocalyx mesh, thus impairing NO release and breaking down a barrier to leucocyte adhesion[103]. Inflammation reduces expression of eNOS or uncouples it from BH₄, so that NO is no longer the product of its reaction and superoxide is produced instead, thus contributing to oxidative stress[104]. Pro-inflammatory mediators such as TNF α and IL-6 can reduce degradation of the natural eNOS inhibitor dimethylarginine, and oxidised LDL increases synthesis of caveolin-1, which inhibits eNOS activation. In addition, the superoxide radical can react with NO to form the reactive nitrogen species peroxynitrite, a highly reactive molecule capable of damaging BH₄[105].

Cell adhesion molecule expression

Upregulation of cell adhesion molecule expression is another central feature of a dysfunctional endothelium. The principal inducible cell adhesion molecules are E-selectin, ICAM-1 and VCAM-1, and they are synthesised and expressed on the luminal EC surface in response to pro-inflammatory mediators such as TNF α and IL-1[87]. If it occurs in arteries, this results in monocyte and T-lymphocyte recruitment into the subintimal space, a critical step in the formation of atherosclerotic fatty streaks[8]. VCAM-1 is particularly important for this process[106]. TNF α binds to its p55 receptor on the EC surface and activates the signalling pathway c-Jun n-terminal kinase (JNK) which then activates NF κ B, the central transcription factor co-ordinating responses to pro-inflammatory stimuli. Activation of NF κ B leads to its translocation to the nucleus and binding directly to consensus sequences in the promoters of E-selectin, ICAM-1 and VCAM-1, resulting in rapid induction of gene expression.

Endothelial cell apoptosis

An increased rate of EC apoptosis is another feature of endothelial dysfunction[107]. EC apoptosis is increased in areas of the vasculature exposed to low shear stress or disturbed flow, which are prone to atherogenesis[108]. EC apoptosis promotes atherosclerosis because denudation of endothelium exposes the basement membrane and thus potentially pro-inflammatory and pro-coagulant factors such as collagen and tissue factor, thus pre-disposing toward thrombosis[109]. SLE patients have increased rates of EC apoptosis[110] and this may reflect reduced VEGF synthesis reported in both the kidney and serum[111].

Endothelial progenitor cell dysfunction

To counteract the effects of EC apoptosis, circulating endothelial progenitor cells (EPC) are capable of instigating endothelial repair, and reduced numbers and function of EPC are also seen in individuals with endothelial dysfunction. EPC are a subset of haemopoietic progenitor cells and originate in the bone marrow. They display some EC characteristics, and are capable of locating sites

of endothelial injury and developing into fully functional EC and influencing repair[112]. Low EPC numbers correlate with a higher incidence of CV events[113].

Both SLE and RA have been associated with reduced numbers of circulating EPC[114]. Grisar *et al* found lower levels of EPC in RA patients with active disease compared to those whose disease was well controlled [115]. Levels of EPC in RA patients have also been shown to correlate with other markers of endothelial dysfunction[116]. In SLE, even patients with inactive disease have reported to have reduced levels of circulating EPC[117], while disease activity correlates with impaired EPC function[118].

1.3.4 Reactive oxygen species

Oxidative stress, an imbalance between reducing and oxidative reactions such that elevated levels of ROS are produced[119], is a feature common to many of the pathophysiological processes seen in endothelial dysfunction[87]. It was once thought to be the defining cause of endothelial dysfunction, atherosclerosis and even of ageing in general, but the lack of success of clinical trials of anti-oxidants suggests this may be over-simplistic.

Over-production of ROS is damaging to cellular components, such as lipids, proteins and nucleic acids, resulting in impairment of normal cell functions. Within EC, elevated ROS can cause a reduction in vascular NO bioavailability, increased expression of redox-sensitive pro-inflammatory genes such as VCAM-1 and, if levels are overwhelming, can precipitate apoptosis[104]. However, oxidative stress also stimulates and regulates many cellular functions including apoptosis, growth, proliferation, genomic stability and vascular tone, and increasing evidence now suggests regulated ROS production within a physiological range is important for intracellular signalling. Therefore a concept is emerging of an optimal range of ROS production required for cellular homeostasis[120].

ROS produced by EC

ROS produced by EC include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and lipid peroxides.

Superoxide is produced by a one electron reduction of oxygen, catalysed by a number of oxidases including NAD(P)H oxidases and xanthine oxidase as well as the mitochondrial electron transport chain[121]. $O_2^{\cdot-}$ in picomolar ranges is metabolised by superoxide dismutase (SOD) into H_2O_2 , a freely diffusable and stable form of ROS which can act as signalling molecule. H_2O_2 can then be removed by conversion to water by catalase or glutathione peroxidase[122]. Whether superoxide and H_2O_2 are harmful depends critically on the amounts produced: while low levels are necessary for signalling and may modulate such responses as smooth muscle cell proliferation, high levels are associated with DNA damage and can lead to EC apoptosis. Examples of H_2O_2 signalling include

acting as a hyperpolarising factor in resistance vessels, activation of guanylate cyclase, stimulation of NO production and altering gene expression[120].

Peroxynitrite is formed by excess superoxide reacting with NO and its production thus leads to a decrease in NO bioavailability as well as increased oxidative stress. This results in impaired endothelium-dependent relaxation. Peroxynitrite is a potent oxidant whose production does not appear to have any useful role in normal EC physiology. Instead it can oxidise lipids and thiol groups on proteins and is an important mediator of LDL oxidative modification[122].

Sources of ROS in EC

The principal sources of ROS within EC are the mitochondrial electron transport chain, NADPH oxidases (Nox enzymes), uncoupled eNOS and xanthine oxidase (XO)[123]. NADPH oxidases and mitochondria are thought to be the major sources under physiological conditions, with XO and eNOS uncoupling producing significant levels of ROS only in pathological states[104].

The mitochondrial electron transport chain represents a significant source of ROS (Figure 1.2). During oxidative phosphorylation, adenosine triphosphate (ATP) is generated as electrons are transferred from nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH₂) to molecular oxygen. This process occurs through a series of electron transport carriers located on the mitochondrial inner membrane: Complex I (NADH-ubiquinone oxoreductase); Complex II (succinate-ubiquinone oxoreductase); Complex III (ubiquinol-cytochrome c reductase); and Complex IV (cytochrome c oxidase). The 5th complex, ATP synthase, uses the gradient formed to generate ATP.

1-4% of the oxygen reacting with the respiratory chain is incompletely metabolised and forms superoxide, which is scavenged by manganese superoxide dismutase (MnSOD) and converted to H₂O₂[124]. Under certain conditions, the electron transport chain can become uncoupled, leading to increased superoxide production. This involves the actions of uncoupling proteins which are discussed later. This may have pathological consequences - for example, causing oxidative damage to mitochondrial DNA - but may also be important for intracellular signalling. Complexes I, II and III can all generate superoxide but only III generates ROS in the intermembrane space to allow signalling to the cytosol[124].

Excess mitochondrial ROS production is harmful: it damages mitochondrial proteins and DNA, promotes inflammation and reduces NO bioavailability. CV risk factors are associated with increased mitochondrial damage in CV tissue, and mitochondrial DNA damage correlates with the extent of atherosclerosis in mouse and human tissue[125]. Mitochondrial ROS have also been linked with Alzheimers dementia and have even been suggested as the cause of ageing[126].

Recent research however suggests that regulated mitochondrial ROS generation may be physiologically desirable in EC[120]. Energy requirements in EC are relatively low and are satisfied by ATP production through glycolysis and so mitochondria do not appear to be needed for ATP generation and may instead function primarily for signalling purposes[124]. Mitochondria make up only 2-8% of EC mass (rather than 30% in cardiac myocytes)[127, 128]. Mitochondrial H₂O₂, produced by MnSOD or Nox 4, can function in physiological signalling as well as being the source of harmful effects. H₂O₂ can play regulatory roles in shear-stress induced vasodilation, autophagy, and inflammatory activation[124]. In myocardial arterioles mitochondria tethered to the cytoskeleton release ROS in response to deformation by shear stress, resulting in NO production[129]. Meanwhile, in rat pulmonary arterial EC, perinuclear mitochondria release ROS that can diffuse to the nucleus and alter expression of hypoxia-sensitive genes[130].

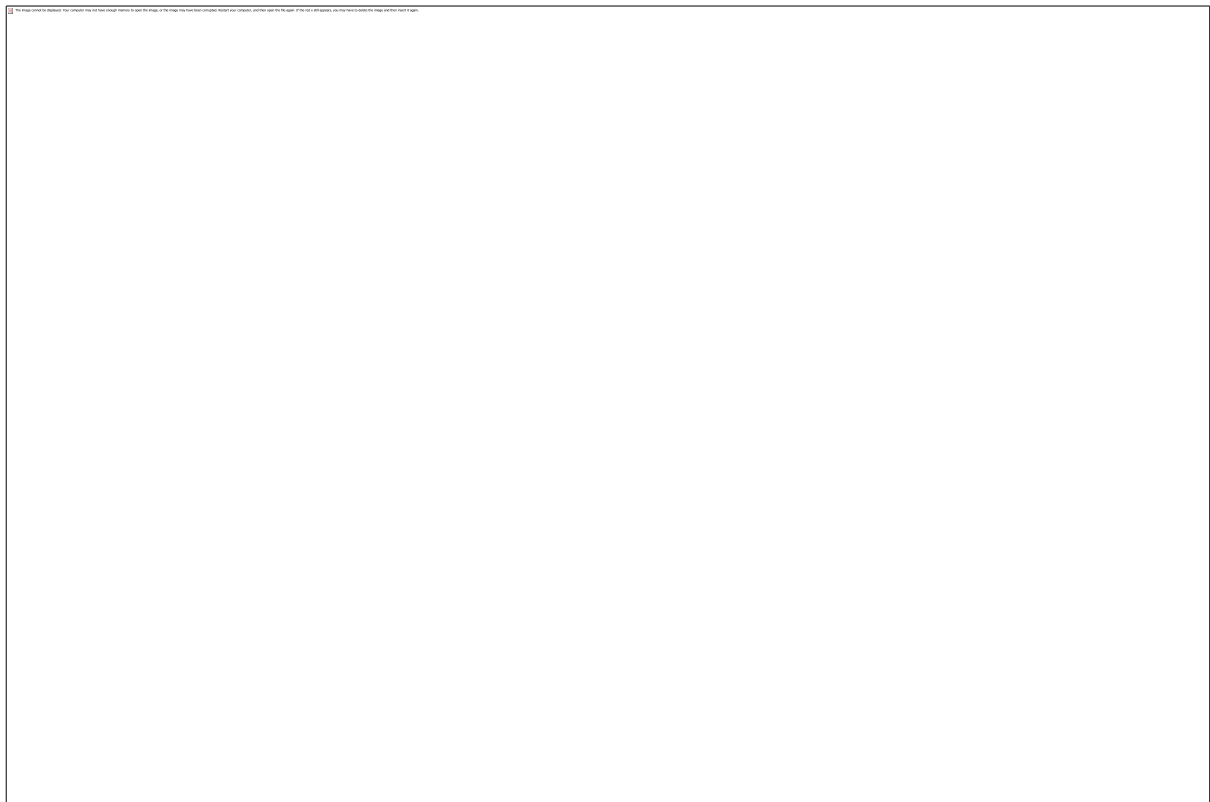


Figure 1.2 Superoxide production and dismutation in mitochondria. Complexes I and III of the electron transport chain can produce superoxide, which is converted to H₂O₂ by MnSOD in the mitochondrial matrix. Further ROS regulation is achieved by the action of uncoupling proteins, which dissipate the proton gradient generated by the electron transport chain across the mitochondrial inner membrane. I-V: electron transport chain; MnSOD: manganese superoxide dismutase; UC: uncoupling proteins.

There are several subtypes of Nox enzyme expressed in EC, in both the cytosolic membranes and within mitochondria. Nox 1 is present at low levels constitutively but is inducible while Nox 4 is most abundant and is constitutively expressed in EC. Nox expression and activity is modulated by vasoactive hormones such as angiotensin II, endothelin-1, by TNF α and by physical forces such as

disturbed flow[104]. Their role in atherogenesis was revealed by studies of Nox deficient mice crossed with ApoE^{-/-} animals, where the double knockouts developed fewer, smaller atheromatous lesions as well as lower levels of aortic superoxide[131].

Uncoupling of eNOS occurs when it is deprived of either its substrate or its cofactor for NO production: L-arginine and BH4 respectively. In this setting, eNOS is unable to transfer electrons to L-arginine and instead uses O₂ as a substrate to produce superoxide. The superoxide can then react with NO to produce peroxynitrite, which oxidises BH4 and thus further uncouples eNOS, reducing NO bioavailability[132]. In studies of the vasculature in the rat adjuvant arthritis model of RA, both Nox enzymes and eNOS uncoupling appeared to play a role in the observed endothelial dysfunction[133].

XO catalyses the conversion of xanthine, an end-product of purine metabolism, and oxygen into uric acid, H₂O₂ and superoxide. XO is induced by pro-inflammatory cytokines including TNF α and by oxidation of cysteine residues by peroxynitrite, and is likely only to be a significant source of ROS in pathophysiological conditions[104]. A study of diet-induced atherosclerosis in hypercholesterolaemic rabbits implicated XO-induced oxidative stress in the disease process[134]; while studies on patients with coronary artery disease demonstrate coronary artery activation of XO as well as NADPH oxidase[135].

Endothelial antioxidant detoxification systems

For there to be a balance between physiological ROS levels for signalling and regulatory purposes and damaging over-production, antioxidant systems exist to metabolise ROS to less reactive molecules. Enzymatic antioxidant systems include SOD, catalase, the glutathione system, the thioredoxin system and NADPH quinone oxoreductase -1 (NQO-1), which catalyse reactions to convert ROS into more stable compounds or to reduce already oxidised proteins. Non-enzymatic systems include ascorbate and α -tocopherol. These generally act as sites to accept or donate electrons in order to stabilise ROS.

Antioxidant defence enzymes are found in specific distributions within cellular compartments: peroxisomes (catalase), mitochondria (SOD, glutathione peroxidase; Figure 1.2) and within the cytosol (SOD, peroxiredoxins, glutathione peroxidase). This selective distribution supports the idea of regional ROS production, which can then signal to other parts of the cell and regulate cellular functions. SODs are discussed in detail later in the chapter.

The major cytosolic system in EC is glutathione, a modified amino acid containing a thiol group. This is present at very high concentrations intracellularly, up to 1mM, and 99% of cytosolic glutathione exists in a reduced state. Glutathione peroxidase catalyses the oxidation of reduced glutathione by H₂O₂ and lipid peroxides and eliminates them. Reduced glutathione is then regenerated via donation of electrons from NADPH, catalysed by glutathione reductase[136].

A further important source of defence against oxidative stress is the thioredoxin (Trx) system, which is involved in the reduction of unwanted disulphide bonds on oxidised proteins in order to maintain the reducing environment of the intracellular space, in conjunction with the glutathione system. They also protect against oxidative and nitrosative stress. Trxs are ubiquitous small proteins found variously in the cytosol, mitochondria, nucleus, embedded in cell membranes and secreted extracellularly. There are two isoforms in mammalian cells: Trx I which is located in the cytosol and in the nucleus, and Trx II, located in mitochondria[137, 138]. Trxs contain two conserved cysteine residues as their catalytic site which break the disulphide bonds on oxidised proteins by passing electrons and forming a disulphide bond with each other. This is then reduced by Trx reductase via NADPH electron donation.

NQO-1 is another cytosolic antioxidant enzyme that uses NADPH as an electron donor. It catalyses the reduction of quinones and thus detoxification. It is also inducible, with transcription controlled by Nrf-2 binding to antioxidant response elements in its promoter. NQO-1's functions in addition to reduction of quinone compounds include prevention of ROS formation and scavenging of superoxide anion radicals[139]. It is highly expressed in human vascular tissue and flow-inducible[140].

1.4 Endothelial cytoprotection

Endothelial cytoprotection refers to the activation of intrinsic protective pathways to counteract the effects of injurious stimuli that predispose to endothelial dysfunction. This results in limitation of vascular inflammation and oxidative stress by anti-inflammatory, anti-oxidant, anti-apoptotic counter regulatory mechanisms that collectively maintain the integrity and homeostasis of the vascular endothelium (Figure 1.3). Studying these pathways is important in order to work out how they might best be enhanced, in order to relieve endothelial dysfunction and thus prevent atherosclerosis.

Endogenous external factors that promote endothelial resistance against injury include laminar flow with high shear stress[11], growth factors such as VEGF and Ang-1, anti-inflammatory cytokines such as TGF β and IL-10, and HDL[141]. Pre-conditioning could also have a significant effect: a brief exposure to oxidant stress or pro-inflammatory cytokines may blunt EC responses to subsequent exposure to injurious stimuli[89].

A number of intracellular signalling pathways and transcription factors have been identified as being important in mediating endothelial cytoprotection. These include extracellular signal-regulated kinase 5 (ERK5), ERK1/2, protein kinase C ϵ (PKC ϵ) and PKC ζ , and AMP-activated kinase (AMPK)[142]. Among the many important transcription factors, there has been recent interest in Kruppel-like factor 2 (KLF2), nuclear factor (erythroid-derived 2)-like factor 2 (Nrf-2), cyclic AMP-responsive element binding protein (CREB), peroxisome-proliferator activated receptor γ (PPAR γ), and in the mechanisms for inhibiting nuclear factor- κ B (NF κ B).

Cytoprotection occurs through the induction of end-effector intracellular proteins and release of vasoactive substances, as well as the down-regulation of pro-inflammatory mediators. These include eNOS and release of NO, heme oxygenase-1 (HO-1), antioxidant enzymes such as SOD and uncoupling proteins and anti-apoptotic and complement-inhibitory proteins.

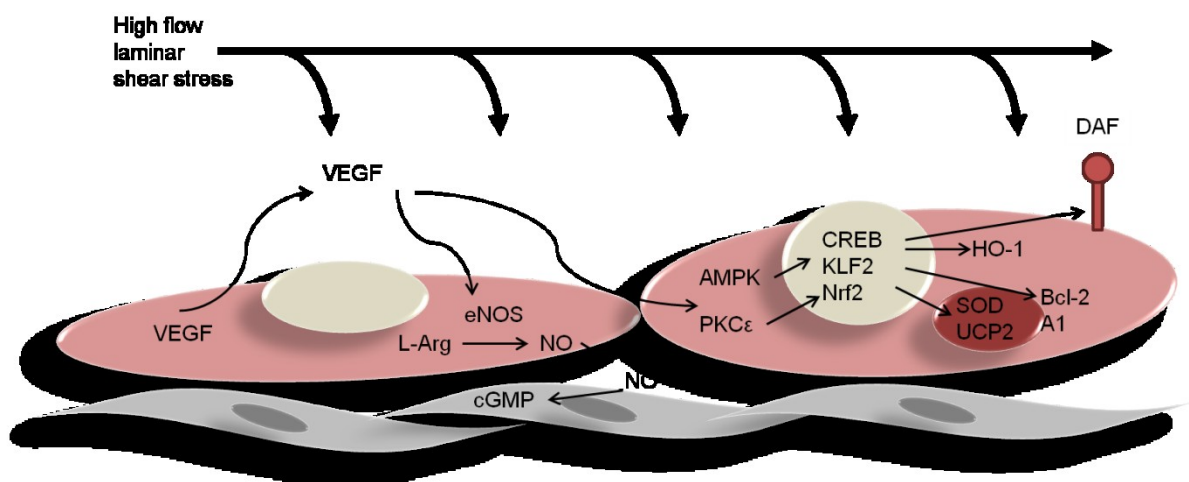


Figure 1.3 Selected mechanisms of endothelial cytoprotection.

1.4.1 High flow laminar shear stress

The geographical distribution of atherosclerotic plaques is powerful evidence that biomechanical forces exerted by blood flow on vessel walls are important in the pathogenesis of atherosclerosis.

Shear stress is the perpendicular force per unit area exerted on a surface (such as endothelium) when it is exposed to a tangential force (such as blood flow). For Poiseuille flow (steady unidirectional laminar flow through a straight cylindrical tube of constant diameter) shear stress is directly proportional to the velocity of blood flow and inversely proportional to the cube of the arterial radius[143]. Pulsatile high flow laminar shear stress (LSS) is therefore experienced by EC situated along straight non-branching arteries, while EC at branch points are exposed to disturbed flow with low shear stress. Post mortem studies of the human carotid bifurcation and the distal internal carotid artery show a predilection for atheroma at the carotid bifurcation, a branch point, and not the internal carotid which is straight[144]. In addition, EC apoptosis, VCAM-1 expression and monocyte adherence, all features of a dysfunctional endothelium, are enhanced in regions exposed to low shear stress[108, 145].

Much research over the last 20 years has been devoted to discovering why LSS protects against atherogenesis, and how this protection is achieved. One initial theory was that of mass transport, which proposed that disturbed blood flow resulted in prolonged exposure of the endothelium to lipids and other pro-atherogenic substances at branch points and curvatures[146]. However, it is now generally accepted that EC sense and react to the flow patterns that they are exposed to, and that shear stress pattern modulates gene expression and thus EC phenotype[143]. Microarray studies have been used to determine the changes in gene expression in EC triggered by different flow patterns. Despite differences in model and environment of the study, these consistently show that high flow LSS downregulates gene expression in EC, promoting a quiescent phenotype resistant to activation[11]. Expression is particularly affected of pro-inflammatory cytokines, adhesion molecules, apoptosis regulators and genes involved in proliferation and migration. Expression of these genes is by contrast increased in areas of disturbed flow and it is this differential regulation of expression that may underpin the localisation of atherogenesis.

Mechanotransduction in EC occurs through a complex of platelet-endothelial cell adhesion molecule (PECAM; CD31), vascular endothelial (VE)-cadherin and VEGF receptor 2 (VEGFR2). PECAM acts as the mechanosensor, and VE-cadherin and VEGFR2, once activated, are able to stimulate a succession of downstream signalling pathways[147]. In EC exposed to high flow LSS, this results in, among a host of responses, enhancement of NO production and priming of anti-inflammatory, anti-apoptotic and anti-oxidant responses.

The primary mechanism by which LSS exerts a protective effect on vascular endothelium is through regulation of NO production, achieved by phosphorylating eNOS on serine 1177, an event dependent

on phosphoinositol-3-kinase (PI-3K)/Akt signalling[148]. PI-3K activation may occur directly following activation of the mechanosensory complex[147].

LSS also promotes an anti-inflammatory phenotype in EC, whereas disturbed flow is pro-inflammatory. Prolonged exposure to laminar flow *in vitro* reduces ICAM-1, VCAM-1 and E-selectin expression, while oscillatory flow upregulates them and enhances monocyte adhesion[149]. Moreover, exposure to IL-1 β in EC conditioned by LSS results in reduced VCAM-1 expression compared to that observed in static conditions[150]. VCAM-1 expression and NF κ B activation are seen *in vivo* in aortic EC located in areas associated with a high probability of atherosclerotic lesion development[151].

Thirdly, LSS is associated in EC with lower levels of oxidative stress. *In vitro*, atheroprotective flow patterns result in lower levels of intracellular ROS and enhanced resistance to external oxidative stress, an effect co-ordinated by Nrf-2 binding to the antioxidant response element (ARE) region of anti-oxidant genes including HO-1, glutathione reductase, NQO-1 and Trx reductase[140]. This may occur through an effect on cytosolic levels of reduced glutathione, which in LSS conditions is enhanced and with disturbed flow is depleted[123]. Hojo *et al* showed that steady laminar flow inhibits JNK activation by H₂O₂, and cell death mediated by TNF α or H₂O₂, partly through an effect on glutathione reductase[152]. In addition, the antioxidant enzymes copper/zinc SOD (Cu/ZnSOD; SOD1) and extracellular SOD (ECSOD; SOD3) are induced by LSS[153], while oscillatory flow is associated with reduced NO production and increased superoxide[154]. Thus, the flow patterns endothelium is exposed to can differentially regulate cellular redox balance.

A further facet of the quiescent phenotype invoked by the action of LSS on EC is inhibition of apoptosis and proliferation. In one recent study, human umbilical vein EC (HUVEC) exposed to LSS for up to three days reduced proliferation by 20% as well as a significant reduction in apoptosis compared to cells cultured under static conditions[155]. Other work has shown that LSS for 24h reduces EC proliferation through p53 expression and stabilisation via JNK-mediated phosphorylation[156]. Finally, shear stress has been shown to inhibit apoptosis induced by growth factor withdrawal, TNF α or H₂O₂[157, 158]

In summary, LSS promotes a quiescent phenotype in EC, characterised by plentiful NO production, reduced oxidative stress, reduced gene expression and slow cell turnover. This state is anti-inflammatory and anti-thrombotic and so atheroprotective.

1.4.2 Vascular endothelial growth factor

The growth factor VEGF-A (VEGF) is a heparin-binding glycoprotein produced by EC and surrounding cells with paracrine effects[89]. It is part of a family of related growth factors, and has at least 5 isoforms ranging from 121 to 206 amino acids in length. The 165 amino acid form is the most biologically active and most abundant form[159].

VEGF is essential in the embryo for vasculogenesis and angiogenesis, and even heterozygous VEGF-deficient mice die *in utero* with a severely mal-developed vasculature[160, 161]. More recent work has shown that it has an important maintenance role for the adult vasculature. Targetted disruption of VEGF in the endothelium of adult mice leads to severe vascular abnormalities[162]. In addition bevacizumab, an anti-VEGF monoclonal antibody used to treat metatstatic cancer and age-related macular degeneration, can cause hypertension and proteinuria in patients, thought to be due to its effects on NO production[163].

VEGF in the adult vasculature has three well-described functions: angiogenesis, EC proliferation and enhancement of vascular permeability [164, 165]. Although studying the role of VEGF in mature vessels *in vivo* is difficult because of its lethality if depleted, *in vitro* work has led to the idea that it has vascular protective functions in mature adult blood vessels, enhancing vasodilative, anti-thrombotic and anti-apoptotic pathways independently of its effects on proliferation[159].

VEGF is required to form and sustain new vessels in adult organisms[164]. This is often a response to pathology, such as angiogenesis in rheumatoid proliferative synovitis, cancer, diabetic proliferative retinopathy, or collateral vessel formation after pathological blood vessel occlusion. Without a VEGF signal new vessels regress, but mature vessels mostly do not require it for survival. However, VEGF is important for the maintenance and function of certain mature vascular beds. For example, it is required to form and maintain fenestrations and so major sites of constitutive VEGF production in mature organs are the cells adjacent to fenestrated endothelium in the glomerulus, the brain choroid plexus and within endocrine organs. The importance of this is shown in a study of lupus nephritis, where loss of VEGF in the kidney correlated with worse renal outcome[111].

The vascular protective actions of VEGF relate to its abilities to enhance NO production, and effects on both prostacyclin synthesis and on EC survival. VEGF enhances eNOS expression and stimulates endothelial NO production through phosphorylation of eNOS[166, 167]. The mechanism for this involves ligation of VEGFR2, a receptor tyrosine kinase and the principal receptor for mediating VEGF intracellular signalling. Evidence that this has vascular protective effects comes from a study of perivascular VEGF gene transfer in a rabbit carotid artery injury model, where addition of VEGF in a carotid artery cuff reduced neointimal hyperplasia, an effect abolished by co-treatment with the eNOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME)[168]. Further support comes from a

study of leucocyte adhesion, where administration of VEGF attenuated thrombin or L-NAME-induced leucocyte adhesion in rat mesenteric endothelium[169].

VEGF also stimulates endothelial production of prostacyclin, another potent vasodilator that also affects on vascular permeability and inhibits platelet aggregation and smooth muscle cell proliferation[170]. As with stimulation of NO synthesis, the mechanism involves ligation of VEGFR2 and then initiation of a signalling cascade involving c-Src, phospholipase C γ 1 and release of intracellular Ca²⁺[171].

The restricted spatial pattern of constitutive VEGF expression in most organs argues against a need for VEGF for mature vessel survival. However, inhibition of VEGF in adult mice caused regression of tracheal capillaries[172] and in fenestrated endothelium sites, although not in the heart and lung[173], and as described above, severe vascular abnormalities were seen in adult mice with a single allele deletion of VEGF, restricted to the vasculature. The effects of VEGF on EC survival may involve VEGF induction of Bcl-2-related protein A1 (A1) and B-cell lymphoma protein-2 (Bcl-2), anti-apoptotic proteins from the Bcl-2 family discussed later in the chapter[174, 175].

Finally, VEGF can protect vascular endothelium against complement-mediated injury, by upregulating cell surface expression of decay-accelerating factor (DAF), a complement inhibitory protein[176, 177]

1.4.3 Pre-conditioning and stress-response hormesis

A further external stimulus for enhancing endothelial cytoprotection is pre-conditioning. When cells are exposed briefly to an adverse stimulus, this can lead to an enhanced ability to withstand a second insult. For example, Zahler *et al* briefly exposed cultured EC to a high concentration of H₂O₂, then stimulated them with TNF α for 4h. Exposure to this powerful oxidative challenge, which was sufficient to reduce the intracellular reduced glutathione concentration by 50%, significantly altered the ability of EC to respond to TNF α , with reduced synthesis and release of IL-6 and IL-8, reduced expression of ICAM-1 and E-selectin, and a reduction in NF κ B p65 translocation. Moreover, these effects were antagonised by pre-treatment with n-acetyl cysteine (NAC), which would preserve the pool of reduced glutathione, showing that the reduction in response to TNF α was due to the oxidative challenge[178].

Pre-conditioning has also been investigated in the field of ageing, where it is described as "stress response hormesis"[179]. Organism and cellular responses to stress are biphasic, with high-intensity or prolonged insults being lethal but low-intensity stressors induce a response that results in stress resistance[89]. This adaptive, defensive response, occurring through alteration in gene expression, improves the ability of the cell or organism to deal with the second stressor. At an organism level, supportive evidence is provided by experiments on *Caenorhabditis elegans*, showing for example that

brief exposure to very high O₂ concentrations extends lifespan[180]. It is possible that a number of activators of AMPK such as metformin, discussed later in the chapter, evolved as mitochondrial poisons as they inhibit the mitochondrial electron transport chain, but at low dose can have beneficial metabolic effects via AMPK activation[181].

1.4.4 Cytoprotective signalling cascades and transcription factors

Many intermediary signalling kinases and transcription factors have been identified as being important in mediating endothelial cytoprotection as a result of activation by LSS, VEGF or other endogenous mediators and only a selection are discussed here. Identifying these pathways allows investigation of how they may be manipulated pharmacologically.

Protein kinase C ϵ

Protein kinase C ϵ (PKC ϵ) is one of the novel isoenzymes of PKC, a family of serine-threonine kinases. It was first discovered as a cell survival factor, and activation results in protection against apoptosis in cardiomyocytes and in EC following ischaemic pre-conditioning[182-184]. Activation of PKC ϵ is an important mediator of the cytoprotective effects of VEGF in EC, mediating protection against apoptosis through induction of A-1 and Bcl-2, and protection against complement-induced injury through induction of DAF[176, 182]. Cytoprotection due to PKC ϵ activity may occur through ERK1/2 and NF κ B activation: PKC ϵ overexpression induces cytoprotective gene expression via NF κ B but not pro-inflammatory genes[175].

Kruppel-like factor 2

Kruppel-like factor 2 (KLF2), one of a family of zinc finger transcription factors, was first identified by transcriptional profiling as being strongly induced by LSS *in vitro*[185]. To date, no other endogenous inducers of its expression have been found, although it is likely also to mediate many of the endothelial protective effects of statins[186, 187]. This, combined with its *in vivo* expression in sites protected against atherogenesis, has led to the suggestion that KLF2 underpins the molecular basis of flow-mediated atheroprotection[143]. Genome wide analyses of gene expression suggest that KLF2 could influence the expression of up to a third of shear responsive genes[188]. It regulates the shear-induced expression of eNOS, thrombomodulin, and CD59, indicating that it is responsible for shear-induced anti-inflammatory, anti-thrombotic, anti-oxidant and complement-inhibitory effects[188-190]. The MEK5-ERK5-MEF2 pathway appears to be the signalling pathway most important for the regulation of KLF2 expression[188, 189].

Regarding the basis for endothelial dysfunction caused by chronic inflammation, it is relevant that KLF2 expression may be reduced by IL-1 β and TNF α . Conversely, over-expression of KLF2 reduces VCAM-1 and E-selectin expression and reduces leucocyte adherence to EC[191, 192].

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are also important for atheroprotection and may mediate some of the beneficial effects of LSS. PPARs are transcription factors belonging to the nuclear receptor superfamily, and there are three subtypes: PPAR α , PPAR β/δ and PPAR γ . PPARs activate transcription by forming heterodimers with the retinoid receptor RXR, and binding to specific PPAR-responsive elements within promoter sequences. Natural ligands of the PPARs are not fully elucidated but include fatty acid derivatives and prostaglandins[141, 193]. However, both PPAR α and PPAR γ are ligands for drugs in clinical use: PPAR α is the site of action of the lipid-lowering drugs fibrates, and the insulin-sensitising drugs thiazolidinediones act on PPAR γ .

Activation of PPAR γ with thiazolidinediones reduces atheroma formation in atherosclerosis-prone mice[194]. *In vitro* studies show that PPAR γ ligands reduce TNF α -induced VCAM-1 expression and thus have anti-inflammatory roles. PPAR α activation can repress cytokine-induced activation of VCAM-1 in EC, and reduce monocyte adhesion[195], and aortae from PPAR $\alpha^{-/-}$ mice display a more exuberant response to pro-inflammatory stimuli, resulting in increased vascular IL-6 production[196]. PPARs also have anti-oxidant actions, and can regulate both catalase and HO-1 expression[197].

PPARs are also capable of acting as transrepressors and inhibiting pro-inflammatory transcriptional mechanisms. For example, PPAR α can negatively interfere with NF κ B and AP-1 transcription, through actions on c-Jun[196].

Nuclear factor (erythroid-derived 2)-like factor 2

Nrf-2 appears to be the transcription factor responsible for mediating the anti-oxidant effects of LSS[140]. This transcription factor binds to the ARE motif, which is found in the promoters of many anti-oxidant genes. Dai *et al* demonstrated, by conducting genome-wide transcriptome profiling on EC exposed to atheroprotective flow patterns with siRNA to Nrf-2, that the major differentially regulated genes were antioxidants including HO-1, ferritin heavy chain, NQO-1, Trx reductase and glutathione reductase. The major upstream kinase in their model was PI-3K/Akt[140]. It may also be possible to manipulate Nrf-2-mediated transcription pharmacologically: Hamdulay *et al* found that Nrf-2 translocation was required for HO-1 induction by celecoxib[198].

Cyclic-AMP Responsive Element Binding Protein (CREB)

CREB is a phosphorylation-dependent transcription factor, part of a family of cAMP-responsive factors that also contains ATF-1 and cAMP response element modulator (CREM) that are all capable of binding to cAMP-responsive elements (CRE) in gene promoter regions and activating transcription. It is responsible for regulating a variety of cellular responses, predominantly cytoprotective in nature. These include cell proliferation, survival, adaptive responses and glucose homeostasis[199].

CREB is activated by phosphorylation on serine 133 in its transactivation domain, and several serine-threonine protein kinases can do this, including AMPK, c-AMPK-dependent protein kinase A (PKA), PKC, particularly PKC ϵ , and calmodulin kinases (CaMKs), which respond to calcium fluxes[199, 200]. Once phosphorylated on Ser133, CREB interacts with the co-activators CREB-binding protein (CBP) or p300, or CREB-regulated transcriptional co-activators (CRTCs), to initiate transcription of genes containing CRE sites in their promoters. This will be discussed in more detail in the introduction to Chapter 6.

There is evidence that CREB activation exerts protective effects on the vasculature. Vascular CREB protein levels are depleted in rodent models of a variety of conditions affecting the vasculature, including hypertension, ageing and insulin resistance[201]. This is partly due to exposure to oxidised LDL and to elevated levels of ROS, as exposure of rat VSMCs to these *in vitro* resulted in loss of CREB protein, and this could be blocked by pre-treatment with ROS scavengers. Second, cardiac-specific expression of dominant-negative CREB leads to a dilated cardiomyopathy with increased mortality and mitochondrial dysfunction in female mice, resulting in survival at 21 weeks of only 17%[202].

Most studies have examined the role of CREB in vascular smooth muscle or cardiomyocytes but it is physiologically important in endothelium too. Exposing HUVEC to oxidised phospholipids results in protective induction of HO-1, mediated by CREB[203]. Depleting CBP by RNA interference sensitises EC to apoptosis by serum starvation[204]. Thirdly, CREB can interact with NF κ B to modulate ICAM-1 expression[205]. Finally, activation of CREB is important for the induction of DAF by atorvastatin and rapamycin combination[206].

The protective effects of CREB activation come from its ability to influence mitochondrial function. Mice harbouring cardiac dominant-negative CREB exhibit decreased PPAR γ coactivator-1 α (PGC-1 α ; a transcriptional co-activator essential for mitochondrial biogenesis) and diminished mitochondrial density and structure. They also have increased ROS levels and reduced mitochondrial antioxidant levels (MnSOD and glutathione peroxidase)[202]. *In vitro* studies confirm that CREB is capable of regulating PGC-1 α expression and thus mitochondrial biogenesis, as well as exerting an anti-apoptotic effect via induction of Bcl-2 and A1, and contributing to antioxidant defence by inducing MnSOD and HO-1[202, 203].

Nuclear factor- κ B

Just as important as those transcription factors activated in cytoprotective pathways is inhibition of NF κ B, the key transcriptional regulator of inflammation. Anti-inflammatory cytokines such as IL-10 and TGF β negatively regulate NF κ B; as do many cytoprotective and anti-inflammatory drugs, including glucocorticoids, and several cytoprotective genes inhibit NF κ B as one of their actions[141].

In its resting state, NFκB is sequestered in the cytoplasm bound to its natural inhibitor, IκBα. Phosphorylation of IκBα by the IκB kinase complex leads to its disengagement from NFκB, followed by its ubiquitination and proteolytic degradation, leaving NFκB free to translocate to the nucleus and initiate transcription[141].

There are several points at which inhibition of NFκB may operate. Firstly, NFκB itself induces synthesis of IκBα, forming a negative feedback loop which limits NFκB activation[141]. Formation of IκB is also enhanced by glucocorticoids. Intracellular redox state is important, as a reducing environment may prevent activation of the IκB kinase complex[207]. Finally, agents which block IκBα degradation and interfere with IκB kinase activation have been investigated as possible anti-inflammatory agents.

1.4.5 Effectors of endothelial cytoprotection

All cells possess endogenous mechanisms to defend against injurious stimuli and limit activated states. Within EC these fall into several broad categories: anti-inflammatory, anti-apoptotic, anti-oxidant, and metabolic changes. In terms of the blood vessel as a whole, vasodilatory and anti-thrombotic actions are also important. Many of the effectors of endothelial cytoprotection discussed below have activities that fall into more than one group.

Nitric Oxide

NO is best known for its powerful vasodilatory effect, which occurs through stimulating production of cGMP to mediate vascular smooth muscle relaxation, and which is the hallmark of a normally functioning endothelium. However, it also acts within EC as a signalling molecule, and as such has physiologically important anti-inflammatory and anti-oxidant actions. NO production is up-regulated by LSS and VEGF, and inhibits leucocyte-endothelial interactions by reducing ICAM-1 and VCAM-1 expression[208]. In *in vivo* models, NO blockade results in enhanced vascular monocyte infiltration and increased cell adhesion molecule and chemokine expression, mainly through effects on NFκB activation. These pro-inflammatory changes are associated with increased vascular superoxide production, and can be reversed with NAC. They can also be inhibited using angiotensin II receptor antagonists[209, 210]. These data suggest that NO is able to counteract the pro-inflammatory and pro-oxidant effects of vasoactive mediators such as angiotensin II.

Heme oxygenase-1

HO-1 is a stress-responsive enzyme that catalyses the rate-limiting step in the degradation of heme into biliverdin (which is then further metabolised to bilirubin by biliverdin reductase), carbon monoxide and iron (which in turn induces expression of heavy chain ferritin). It is present at low levels in quiescent cells but is highly inducible, and has been shown in a variety of settings to have protective properties that promote restoration of homeostasis, in both the cell and the whole

organism[211]. The products of its reaction are anti-oxidant and anti-inflammatory and can additionally be involved in the regulation of cell growth and death. Its protective effects *in vivo* are demonstrated by the poor response to stressors shown by *Hmox*^{-/-} mice: these are much more likely to die when challenged with endotoxic shock or autoimmune neuroinflammation[212, 213]; while induction of HO-1 protects against nephritis in murine lupus and reduces synovial inflammation[214, 215].

In vascular endothelium, HO-1 induction exerts anti-apoptotic, anti-inflammatory and anti-oxidant effects that collectively result in endothelial cytoprotection and protection against atherogenesis. Induction of HO-1 is responsible for the protection against oxidative damage provided by atorvastatin treatment in EC, an effect dependent on KLF-2[186]. HO-1 is involved in the inducible cell-surface expression of DAF, and loss of HO-1 results in enhanced susceptibility to complement-mediated injury in EC[216]. HO-1 induction is associated with protection against apoptosis induced by TNF α and ceramide, through AMPK signalling and activation of HO-1 expression by Nrf2 binding to ARE sequences in its promoter[217]. Consistent with the distribution of atherosclerotic plaque in the vasculature, HO-1 is strongly induced by "atheroprotective" flow patterns, via activation of PI-3K/Akt and Nrf2[140]. HO-1 induction also retards the development of atherosclerosis in LDLR^{-/-} mice and is associated with stabilisation of vulnerable plaque in humans[218, 219]. Finally, in the only known case of human homozygous HO-1 deficiency, oxidative stress, extensive vascular endothelial damage and death in childhood was observed[220].

Manganese superoxide dismutase

Superoxide dismutase enzymes catalyse the conversion of superoxide to the more stable hydrogen peroxide. There are three isoforms of superoxide dismutase in the human genome: Cu/ZnSOD located in the cytoplasm, MnSOD, located in mitochondria, and ECSOD. SOD1 and SOD3 share substantial sequence homology but MnSOD differs significantly[221]. MnSOD is most pertinent to the work presented in this thesis and will therefore be the focus of this section.

MnSOD is a 222 amino acid protein, including a 24 amino acid leader peptide that is necessary for mitochondrial targeting and is then cleaved to leave the active enzyme. It is located intracellularly within the mitochondrial matrix, where it scavenges superoxide produced by the electron transport chain. It is one of the major defences against mitochondrial-derived ROS, and control of MnSOD activity and expression levels is a major mechanism of control of signalling via mitochondrial ROS.

MnSOD is induced by a wide variety of cellular stimuli. These include pro-inflammatory cytokines, including TNF α , IL-1 and IFN γ [222, 223], and other toxic stimuli such as ionising radiation.

Induction of MnSOD under these circumstances is principally mediated via activation of NF κ B[224].

The importance of MnSOD for vascular health was first shown in studies of MnSOD deficient mice, which exhibited a dilated cardiomyopathy and 100% neonatal lethality at 10 days. Although the

authors suspected that this was due to mitochondrial dysfunction, given the importance of mitochondria to cardiomyocytes and the subcellular localisation of MnSOD, they could not demonstrate a structural abnormality by electron microscopy[225]. Further evidence of MnSOD's importance to the vasculature comes from studies showing that ApoE^{-/-} mice also deficient in SOD2 developed increased mitochondrial DNA damage and accelerated atherogenesis at arterial branch points[226], while heterozygous MnSOD deficient mice have impaired endothelium-dependent vasodilation[227]. Although some studies have found Cu/ZnSOD to be regulated by laminar flow, this has not been demonstrated for MnSOD, suggesting that, although it is cytoprotective, it is not part of the group of genes whose expression is regulated by Nrf2[140].

In contrast, it is not clear if Cu/ZnSOD operates as a cytoprotective gene in the vasculature. Tribble *et al* showed that in mice overexpressing human Cu/Zn SOD, atherogenesis increased in direct relation to aortic Cu/ZnSOD activity[228].

BCL-2 family

The BCL-2 family of proteins are involved in the regulation of the intrinsic or mitochondrial apoptosis pathway. There are approximately 12 family members in mammals, with either anti-apoptotic (BCL-2, A1, BCL-XL) or pro-apoptotic (BAX, BAK) actions. BCL-2 itself was first discovered in B-cell follicular lymphomas as an oncogene, as its over-expression inhibits cell death[229]. They are essential for the regulation of cell death in normal and pathological tissue turnover, and for immune system activation and deactivation.

The mitochondrial pathway of apoptosis is that which controls cell death in response to developmental or cytotoxic cues such as viral infection, DNA damage or growth factor deprivation. It is strictly controlled by the BCL-2 family, and leads to the activation via cytochrome *c* and apoptotic protease activating factor of caspase 9. The pro-apoptotic members BAX and BAK induce permeabilisation of the outer mitochondrial membrane, leading to release of proteins that normally reside in the inter-membrane space that promote apoptosis such as cytochrome *c*, which then initiate caspase activation. They also induce fragmentation of mitochondria. BCL-2, A1 and BCL-XL inhibit BAX and BAK, although exactly how they do so is not understood. Regulation of their levels, however, is an important way by which cells determine whether apoptosis proceeds[230].

BCL-2 and A1 are important for the regulation of EC apoptosis. Increased EC apoptosis is a feature of endothelial dysfunction in a number of settings, notably SLE. Induction of BCL-2 by a constitutively active PKC ϵ virus protects against EC apoptosis[182]. BCL-2 and A1 may also act to limit inflammatory activation. A1 is rapidly induced by the pro-inflammatory cytokines TNF α and IL-1 β [231], as well as by growth factors such as VEGF[175]. Their importance is shown by *in vivo* experiments involving xenografts: EC in hamster hearts transplanted into rats are rejected if they do

not express BCL-2. In contrast, BCL-2 over-expression inhibits VCAM-1 and E-selectin expression in EC, through inhibition of NF κ B[232].

Complement inhibitory proteins

Complement activation may be important in the development and perpetuation of atherosclerosis as complement cascade proteins, such as C1q, C3a, C5a and the C5b-9 membrane attack complex (MAC), are capable of activating EC, inducing expression of cell adhesion molecules and cytokines, increasing leucocyte adhesion and generating a prothrombotic surface[233-236]. To combat this, EC express inducible complement inhibitory proteins on the cell surface. DAF regulates complement activation by preventing the formation and accelerating the decay of C3 and C5 convertases, thus interrupting the complement cascade and reducing MAC formation. DAF is induced by VEGF, via a mechanism that involves ligation of VEGFR2 and PKC ϵ [176, 177]. It can also be induced by statins, particularly atorvastatin in combination with rapamycin[206, 237]. The mechanism involves activation of AMPK and CREB as well as HO-1[206, 216]. CD59, another complement inhibitory protein, acts by preventing C9 incorporation into the MAC, and is induced by LSS[189]. The clinical importance of complement inhibitory proteins is evident in the disease paroxysmal nocturnal haemoglobinuria, caused by mutations in the *PIGA* gene that results in loss of DAF and CD59 expression.

Uncoupling proteins

Uncoupling proteins are mitochondrial proteins that uncouple ATP generation from oxidative phosphorylation. They are located on the mitochondrial inner membrane and act by dissipating the proton gradient generated by the cytochrome enzyme complexes thus making the membrane potential less negative and reducing ATP production by the electron transport chain. This action means they also reduce levels of mitochondrial ROS production, along with other inducible anti-oxidant enzymes, and may therefore be important for the regulation of ROS for signalling purposes[238].

Three uncoupling proteins have been identified in the human genome: uncoupling protein-2 (UCP2) is the primary isoform in EC. Epidemiological evidence that UCP2 is important for cardiovascular health comes from studies showing that SNPs associated with increased UCP2 expression are also associated with reduced cardiovascular events[239]. Furthermore, UCP2^{-/-} mice exhibit increased atherosclerosis and impaired endothelium-dependent vasodilation[240]. In EC, UCP2 is important for control of mitochondrial superoxide production, for signalling purposes in response to growth stimulation, and its absence may lead to cell senescence[241].

1.5 Therapeutic conditioning of the vascular endothelium

Endothelial dysfunction is the initial stage in atherogenesis. It is also reversible. Exercise, weight loss, dietary and lifestyle modifications, and drugs or combinations of drugs are all capable of improving functional markers of endothelial function, at least in the short term. In the setting of chronic inflammatory disease, if a clear pharmacological strategy could be identified to prevent or reverse endothelial dysfunction, it might be possible to reduce substantially progression to plaque formation. Identification of such a path for therapeutic conditioning of the endothelium is the main aim of the work presented in this thesis.

A number of drugs can improve markers of endothelial function. In addition to statins which are the most widely studied, PPAR γ and PPAR α agonists and metformin also exert beneficial effects. Although these are in clinical use as cholesterol-lowering, anti-hypertensive and anti-diabetic drugs respectively, they have pleiotropic actions; and may act directly on the vascular endothelium in beneficial ways. The actions of metformin and others in activating AMPK will be discussed in the next section.

Statin therapy was first shown to improve endothelial function in patients with hypercholesterolaemia in 1994, comparing pravastatin 10-20mg daily for 6 months with placebo[242]. Subsequent studies have shown that statins improve endothelial function very rapidly, with even short-term courses associated with improvements, and this is not necessarily dependent on the degree of cholesterol reduction[243]. The likely mechanism relates to increased endothelial NO production, principally via increasing eNOS availability and activity, as giving the eNOS inhibitor L-NMMA alongside statins blocks their effect on endothelial-dependent vasodilatation[244]. *In vitro* studies indicate that LDL can increase the inhibitory interaction between caveolin-1 and eNOS, and that atorvastatin decreases caveolin-1 expression and increases the eNOS and Hsp90 interaction[245]. In addition, statins activate Akt, which then phosphorylates and activates eNOS[246]. Lastly, statins may have an anti-inflammatory effect on endothelium, as their use is associated with reduced levels of circulating P-selectin and ICAM-1, and they also induce expression of cytoprotective genes such as HO-1 and DAF[237, 247, 248].

Drugs activating PPAR α and PPAR γ are in clinical use as lipid-lowering and anti-diabetic medications respectively, although PPAR γ agonists are treated with caution as there is evidence of cardiotoxicity with their use and one, rosiglitazone, has been withdrawn[249]. However, multiple lines of clinical, *in vivo* and *in vitro* evidence suggest that they may be a good pharmacological means of stimulating endothelial cytoprotection. Gemfibrozil, a PPAR α ligand, was shown in the Veterans' Administration HDL Intervention Trial to reduce atherosclerotic events by more than would be expected by the observed reduction in LDL concentration[250]. Both PPAR α and PPAR γ ligands can

induce HO-1 and enhance endothelial cytoprotection, while the PPAR α ligand fenofibrate can improve EC survival through AMPK activation[197, 251].

In RA, treatment with anti-TNF α can improve endothelial function as measured by flow-mediated dilatation[252], and can increase numbers of EPCs[253]. DMARDs can also reverse endothelial dysfunction[96], and so can rituximab[254]. In addition, other drugs known to be beneficial to the vasculature may also improve endothelial function in RA, including statins[255] and ACE inhibitors[256].

However, not all drugs in rheumatology practice have benign effects on the endothelium. COX-2 inhibitors and NSAIDs may have damaging effects as a number have been linked with increased risk of MI, although it should be noted that this is not a universal class effect, and indeed some may be beneficial to the endothelium: for example, celecoxib can induce HO-1, at least *in vitro*[198, 257]. In addition ciclosporin may worsen endothelial function and pre-dispose to hypertension, although this has largely been studied in transplant patients[258]. However, *in vitro* studies have shown that ciclosporin antagonises the beneficial action of VEGF in inducing DAF expression, and also induces expression of the vasoconstrictor endothelin-1[176, 259]. This underscores the need for detailed knowledge of which anti-rheumatic drugs improve endothelial function, and how they do it.

1.6 AMP-activated kinase

1.6.1 Structure of AMPK and regulation of activation

AMPK is a ubiquitous serine/threonine signalling kinase originally discovered as a sensor of cellular metabolic status that responds to changes in the intracellular ratio of AMP: ATP[260]. It is conserved throughout all eukaryotes, and its function, and that of its homologue SNF1 in unicellular organisms such as yeast, is to co-ordinate the response to nutrient deprivation. When the ambient glucose concentration is low, AMPK and SNF1 activation leads to switching off of anabolic processes such as protein biosynthesis, cell growth and proliferation, and promotion of efficient generation of ATP through mitochondria rather than relying on glycolysis. In multicellular animals with hormonal and metabolic systems for controlling serum glucose concentrations, intracellular glucose deprivation is rarely experienced and the AMPK system has been adapted to respond to other stressors that disrupt homeostasis such as, in EC, pro-inflammatory cytokines, toxins and oxidative stress[261].

Structure

The AMPK molecule is a heterotrimeric complex composed of a catalytic α subunit, scaffolding β and regulatory γ subunits. There are two or three genes encoding each subunit in the human genome and thus twelve possible AMPK complexes.

There are two α subunit isoforms, $\alpha 1$ and $\alpha 2$, both of which are present in EC, although $\alpha 1$ is much the more abundant[142, 262]. Both contain a phosphorylation site, threonine 172, that markedly enhances their catalytic activity, in addition to the kinase domain at their N-terminal end[263]. They also have slightly different distributions, with $\alpha 1$ being predominantly located in the cytoplasm, while $\alpha 2$ can translocate to the nucleus, suggesting it may be more important in the control of gene expression. $\alpha 2$ is also thought to be more sensitive to AMP binding than $\alpha 1$ [264].

The γ subunit contains three sites, known as Bateman domains, for binding adenine nucleotides and which can bind AMP, two of which can also bind adenosine diphosphate (ADP) or ATP[265]. AMP or ADP binding promotes Thr172 phosphorylation in the α subunit, while ATP binding does not[181].

The β subunits predominantly act as a bridge linking α and γ , but also contain a sequence for binding glycogen. The significance of this is not entirely clear, but it may be important for localising the AMPK complex with glycogen and with enzymes involved in glycogen synthesis and catabolism, which are targets for AMPK activity[181].

Mechanism of activation

AMPK activation occurs through two different mechanisms: binding of AMP to the γ subunit, which increases activity about 5 fold, and phosphorylation at Thr172 in the α subunit, which increases activity approximately 100 fold[266, 267]. AMP binding to the three sites in the γ subunit results in a conformational change that renders Thr172 a better substrate for its upstream kinases. It also allosterically activates the enzyme and slows dephosphorylation by the protein serine/threonine phosphatases PP2a and PP2c[268]. Recent studies suggest that ADP also binds to the γ subunit, protects against dephosphorylation, and may promote phosphorylation[269]. ATP can also bind to the γ subunit, and this antagonises the effect of AMP.

The upstream kinase responsible for Thr172 phosphorylation is liver kinase B1 (LKB1), a tumour suppressor gene that is the site of the mutation causing Peutz-Jeghers syndrome[266, 270]. LKB1 itself is constitutively active and would permanently activate AMPK without a control mechanism. Therefore, it is AMP or ADP binding, rendering the AMPK complex receptive to Thr172 phosphorylation and preventing dephosphorylation, that determines whether activation of the pathway occurs[267].

There is a third mechanism of AMPK activation, which was discovered in neurones and tumour cell lines (such as the cervical cancer HeLa cell line) which lack LKB1. In these cells, AMPK activation can still occur, through increases in cytosolic Ca^{2+} concentrations, which result in AMPK phosphorylation by Ca^{2+} /calmodulin-dependent kinase kinase- β (CaMKK β)[271, 272]. This may be an important mechanism of AMPK phosphorylation in EC, where a number of stimuli, such as bradykinin and thrombin, are capable of causing rapid changes in intracellular calcium and equally rapid changes in AMPK activation state[273].

Regulation of activation by metabolic factors, hormones and cytokines

Any metabolic stress that inhibits ATP production or increases its consumption will activate AMPK. Example of this include glucose deprivation or exposure to the glucose analogue 2-deoxyglucose, which depletes ATP through conversion to the non-metabolisable 2-deoxyglucose-6-phosphate, thus preventing generation of ATP through glycolysis or oxidative phosphorylation[261]. This is principally a research tool: physiologically, mammalian cells only stop producing ATP from glucose when glucose concentrations drop to profoundly low levels. However, more modest degrees of hypoglycaemia also activate AMPK in EC *in vitro*[274].

Other stressors that interfere with oxidative phosphorylation also activate AMPK: for example hypoxia, ischaemia and poisons such as arsenite and dinitrophenol[275-277]. More physiologically, skeletal muscle contraction uses ATP and thus activates AMPK, and many of the desirable metabolic

effects of exercise may be mediated through AMPK signalling[278, 279]. Furthermore, although glucose deprivation is not usually a signal for AMPK activation in mammalian cells, it is used within the physiological range of glucose in certain specialised tissues: for example the β cells in the pancreatic islets of Langerhans responsible for insulin secretion; and in the hypothalamus, where AMPK activation appears to play a role in moderating feeding behaviour[280, 281].

Several hormones that operate through G-protein coupled receptors signal via AMPK, including adrenaline via $\alpha 1$ adrenoceptors in adipocytes, bradykinin in bovine aortic EC and thrombin in HUVEC[273, 282, 283]. These are all likely to activate AMPK via release of intracellular calcium and thus CaMKK β -mediated phosphorylation[284]. In addition, the adipokines leptin and adiponectin activate AMPK $\alpha 2$ in skeletal muscle, where they stimulate glucose uptake and fatty acid oxidation[285, 286]. In the liver, adiponectin also activates AMPK, where the result is a reduction in gluconeogenesis[286].

1.6.2 Pharmacological activators of AMPK

Experimentally, activation of AMPK is often achieved by treating cells or animals with AICAR, an adenosine analogue. AICAR is taken up by cells via adenosine transporters, then converted by adenosine kinase into the phosphorylated nucleotide 5-aminoimidazole-4-carboxamide ribonucleoside (ZMP), which mimics the effects of AMP on AMPK activation[287].

Metformin, a widely prescribed drug used to improve insulin sensitivity in type II diabetes mellitus, activates AMPK *in vitro* and *in vivo*[288]. It is activation of AMPK in the liver, leading to a reduction in hepatic gluconeogenesis, that is most likely responsible for its clinical efficacy[289]. Although metformin has been used *in vitro* to activate AMPK in other tissues, including EC, the concentrations required are not achievable in patients other than in the portal venous system and liver, and thus it is unclear whether metformin activates AMPK in EC in patients [261, 262]. The mechanism by which metformin activates AMPK is thought to be through inhibition of complex I in the mitochondrial electron transport chain, leading to a change in the AMP:ATP ratio and thus allosteric activation[288].

Atorvastatin and simvastatin activate AMPK in EC and in the vasculature *in vivo*[290, 291], and this may be an important contributor to the pleiotropic vasculoprotective properties of statins. Although the mechanism is not clear, AMPK activation is linked to inhibition of HMG CoA reductase, which is the primary effect of statins, and both can increase vascular eNOS activity[245, 292, 293]. Statins may also alter LKB1 phosphorylation, through activation of PKC ζ [291].

PPAR α and γ agonists have also been used to activate AMPK *in vitro*, but there is as yet little evidence to suggest that this is an important facet of their mechanism of action *in vivo*. Fenofibrate, a PPAR α agonist in clinical use to treat hyperlipidaemia, prevented apoptosis induced by serum deprivation in retinal EC through activation of AMPK and induction of VEGF mRNA[251]. This

effect appeared to be PPAR independent. More recently, it was demonstrated that rosiglitazone, a PPAR γ agonist, can activate AMPK and thus have effects on NO production in EC[294].

It was recently discovered that salicylate can directly activate AMPK[295]. Salicylate is the active aspirin metabolite responsible for the majority of the pharmacological effects attributed to aspirin. Activation of AMPK by salicylate may be important for the anti-cancer, as well as anti-inflammatory, properties of aspirin, although the concentrations used *in vitro* are probably not achievable in patients taking conventional low dose aspirin for prevention of coronary artery disease[296]. For example, in order to demonstrate increased acetyl CoA carboxylase (ACC) phosphorylation in response to salicylate (a key AMPK target critical for its effects on fatty acid oxidation), Hawley *et al* used doses sufficient to generate plasma concentrations of 1-2mM in mice[295].

A wide variety of other drugs and herbal supplements have also been shown to activate AMPK, including resveratrol (found in red wine), epigallocatechin gallate (green tea), capsaicin (chillis) and curcumin (turmeric)[297-299]. All act in some sense as a mitochondrial poison, inhibiting the respiratory chain or ATP synthase and thus causing AMP or ADP concentrations to rise. *In vitro* work has demonstrated cytoprotective properties for all these compounds[181].

1.6.3 Targets and metabolic effects of AMPK activation

A wide array of cellular processes are now known to be regulated by AMPK. These include carbohydrate and lipid metabolism; promotion of mitochondrial metabolism and biogenesis; autophagy; cell growth and proliferation; apoptosis; and anti-inflammatory pathways. In many cases AMPK directly targets and phosphorylates effector enzymes in the metabolic pathways under influence. This is the case with ACC, HMG CoA reductase and eNOS[260, 292, 293]. In other cases, AMPK activates transcription factors and co-activators to alter gene expression[142]. Finally, AMPK activation may have target effects via epigenetic regulation: AMPK has been reported to affect the action of histone acetylases and deacetylases, including sirtuin-1 (Sirt1)[300].

Metabolic effects

AMPK is thought to operate as a sensor of cellular energy depletion that, when activated by a rise in AMP:ATP ratio, switches on catabolic pathways capable of generating ATP and turns off energy-requiring processes that are not essential for cell survival. These changes have been explored most extensively in skeletal and cardiac muscle, and in the liver. The catabolic pathways affected by AMPK include upregulation of glucose transporter expression and activity, leading to increased cellular glucose uptake[301]; increased glycolysis via increased activity of 6-phosphofructo-2-kinase[302]; increased fatty acid oxidation[303]; and autophagy[304]. The anabolic processes that are suppressed include glycogen synthesis and hepatic gluconeogenesis[305, 306]; fatty acid and cholesterol synthesis (via inhibition of HMG CoA reductase)[293, 307]; and protein synthesis (via

suppression of MTORC1)[308]. These changes occur both through direct actions on target enzymes and indirectly through activation or inhibition of transcription factors and co-activators and changes in gene expression.

Mitochondrial metabolism

A significant change in cell metabolism promoted by AMPK is increased mitochondrial oxidative metabolism: both by increasing expression of enzymes involved in oxidative phosphorylation, and by inducing mitochondrial biogenesis[266]. Mitochondrial biogenesis is a well-defined feature of activation of AMPK signalling in EC, and in energy-requiring tissues is orchestrated via the transcriptional co-activator PGC-1 α [309].

In HUVEC, AMPK activation using metformin or AICAR resulted in induction of PGC-1 α , stimulated mitochondrial biogenesis, and increased mRNA expression of nuclear respiratory factor-1 and mitochondrial DNA transcription factor A[262, 310]. These effects were associated with a reduction in mitochondrial ROS production.

However, in EC, mitochondria are not the primary energy source; rather they rely mainly on glycolysis[124]. It is therefore not clear what the physiological function of enhanced mitochondrial biogenesis might be, if not to do with a response to increased ATP requirements. It has recently been proposed that mitochondrial biogenesis and PGC-1 α activation in EC may be more important for orchestrating the cellular defence against oxidative stress, through the induction of mitochondrial antioxidants[262]. It is also possible that endothelial function depends on the whole process of mitochondrial turnover, both biogenesis and increased mitophagy/ autophagy, which is also regulated by AMPK[124].

Cell growth and proliferation

Given that the principal stimulus to AMPK activation is nutrient deprivation, it is unsurprising that a further effect of AMPK activation is to inhibit cell growth and proliferation, in both normal and malignant cells. This is likely to be important in its anti-cancer effects. AMPK switches off the mammalian target of rapamycin (mTOR) - S6 kinase I pathway that is critical for regulation of cell growth and size, via phosphorylation of the upstream regulator tuberous sclerosis complex-2 (TSC2) [311]. Inhibition of mTOR results in the downregulation of proteins required for rapid cell growth, including the transcription factor hypoxia inducible factor 1 α (HIF-1 α)[312].

In addition, AMPK activation induces cell cycle arrest in G1, inhibiting transition to S-phase. This state is associated with accumulation of p53 and of the cyclin-dependent kinase inhibitors p21 and p27, which act downstream of p53[313].

Apoptosis, senescence and lifespan

The effect of AMPK on apoptosis is not uniform across all tissues. In most cases in normal, non-activated cells, AMPK activation acts to protect against apoptosis[217, 277]. However, in malignant cells, AMPK activation may promote cell death[314, 315].

AMPK activation may also contribute to cell senescence. Activation of AMPK and an increased AMP:ATP ratio are associated with senescence in fibroblasts after a high number of passages in culture[316].

There is also evidence that AMPK orthologue signalling is important for senescence and thus lifespan in the nematode worm *Caenorhabditis elegans*. *C. elegans* senescence is associated with increases in cellular AMP:ATP. Environmental stresses such as starvation or heat shock that cause rises in AMP:ATP or mutations inactivating the *C.elegans* equivalent of the insulin/ IGF-1 pathway (that antagonises AMPK in mammalian tissues) can only extend the lifespan of worms expressing the full AMPK orthologue[317]. Furthermore, glucose restriction extends *C.elegans* lifespan through the induction of mitochondrial oxidative metabolism, an effect dependent on AMPK activation and sirtuins[318]. This raises the possibility that AMPK activation is implicated in the increase in life span induced by caloric restriction in mammals. Although direct evidence of a role for AMPK signalling in delaying ageing in humans is missing, there is evidence that long-term physical exercise improves mitochondrial metabolism and reduces the decline in mitochondrial function with age, and similar changes in mitochondrial metabolism have been attributed to AMPK activation *in vitro* and in rodents[262, 279, 319].

Anti-inflammatory effects

There is increasingly good evidence that activation of AMPK results in limitation of inflammation. Partly this is due to its effects on cell metabolism: activated cells of the immune system rely on glycolysis for energy; whereas quiescent cells use primarily oxidative phosphorylation in mitochondria[266]. AMPK signalling favours mitochondrial metabolism and so tends to direct cells towards a more quiescent phenotype. For example, in dendritic cells, the switch to aerobic glycolysis following activation by ligation of toll-like receptors (TLRs) is associated with reduced AMPK Thr172 phosphorylation and can be inhibited by pharmacological activation of AMPK[320]. In macrophages, the pro-inflammatory M1 phenotype is associated with aerobic glycolysis while the anti-inflammatory M2 phenotype uses more oxidative phosphorylation[321]. Studies of macrophages depleted of AMPK suggest that it normally lowers the expression of pro-inflammatory cytokines in response to lipopolysaccharide (LPS)[322]. Meanwhile, in mice with a genetic deletion of the AMPK β 1 subunit, macrophages demonstrated increased JNK activation and hence a predominant M1 phenotype[323].

In addition, AMPK signalling is able to invoke anti-inflammatory effects through inhibition of NFκB signalling, and these actions are discussed in the later section on AMPK in EC.

1.6.4 Actions of AMPK in EC

In EC, AMPK activity has similar effects on metabolism as those described in other tissues, including effects on glucose and lipid metabolism and on mitochondrial biogenesis and function (Figure 1.4)[142]. However, whether AMPK has as important a role in EC metabolism as it does in other tissues is less clear, particularly as EC are thought to rely on glycolysis with relatively little energy derived from glucose oxidation[124].

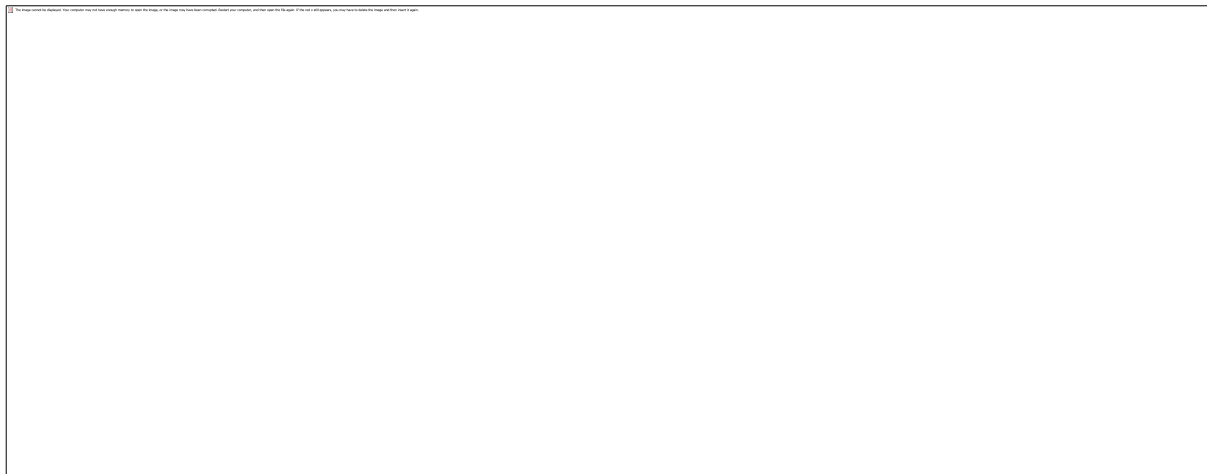


Figure 1.4 Targets and physiological consequences of AMPK activation in EC. Adapted from Fisslthaler and Fleming[142].

In vitro, as in other cell lines, AMPK can be activated in EC via AICAR, glucose deprivation, hypoxia and pharmacologically, resulting in diverse cytoprotective effects[262, 276]. Additionally, and more relevant to the natural physiological state, stimuli specific to EC such as LSS and VEGF activate it[290, 324].

The mechanism by which LSS leads to activation is not clear, but it appears to differ from that by which LSS leads to the phosphorylation of other shear-responsive pathways such as Akt. The evidence for this comes from deletion of CD31, which forms part of the mechanotransduction complex in EC. This did not alter AMPK phosphorylation in response to shear stress[325]. One of the most important protective effects of AMPK in EC is direct phosphorylation of eNOS at both Serine 1177 and Serine 633, leading to increased endothelial NO production [292] and this is likely to be closely linked to its activation by shear stress, given that blood flow powerfully activates eNOS. In addition, the forkhead transcription factor FoxO1a depends on AMPK for its phosphorylation by flow, leading to its export out of the nucleus and downregulation of expression, in turn leading to a reduction in HMG-CoA reductase and Ang-2 expression[326]. Lastly, flow-induced AMPK signalling may be important for the induction of KLF-2, as Young *et al* reported that blocking AMPK resulted in

abrogation of the flow-mediated induction of KLF-2 expression. This effect depended on downstream changes in ERK5/ MEF2 signalling[327].

Secondly, AMPK may be important for angiogenesis. Embryonic LKB1 knockouts die *in utero* with vascular abnormalities[328], and dominant negative AMPK virus infection of HUVEC inhibited their migration along a VEGF gradient and angiogenesis in *in vitro* and *in vivo* assays[329]. This effect may be mediated through AMPK α 1, as inhibition with RNA interference reduced VEGF-induced angiogenesis in matrigel plug assays[324]. Of note, although VEGF is also a powerful stimulator of NO formation by eNOS in EC, this appears independent of its activation of AMPK, as inhibition of AMPK or its upstream kinase CaMKK β did not affect VEGF-mediated eNOS phosphorylation[324].

Thirdly, AMPK activation in EC can protect against oxidative injury, through its effects on mitochondrial function and biogenesis. EC pretreated with AICAR or metformin were protected against injury and death induced by rising concentrations of H₂O₂. This effect involved abrogation of JNK pathway activity, reversal of changes in mitochondrial membrane potential and mitochondrial ROS production induced by H₂O₂, and enhanced mitochondrial biogenesis[262]. It also involved the transcriptional co-activator PGC-1 α , which in muscle is a critical controller of mitochondrial metabolism through AMPK[330].

Part of the explanation for how AMPK protects against oxidative injury presumably derives from its ability to induce expression of anti-oxidant genes. Silencing AMPK α 1 in human EC resulted in decreased expression of many anti-oxidant genes, including MnSOD, catalase, γ -glutamyl cysteine synthase and Trx[276]. These changes required PGC-1 α , CREB and FoxO3a. AMPK α 2 is important too however, despite being expressed at lower levels in EC. Wang *et al* demonstrated impaired endothelium- dependent relaxation in response to acetylcholine in aortae from AMPK α 2^{-/-} mice, which was improved by pre-treatment with a SOD mimetic, suggesting that increased oxidative stress was responsible. They also noted increased expression of NADPH oxidase subunits and superoxide production in HUVEC treated with DN-AMPK or AMPK α 2 siRNA[331].

Fourthly, AMPK activation acts to prevent apoptosis induced by a variety of stimuli including TNF α , hyperglycaemia and oxygen and glucose deprivation[217, 277, 332]. When EC are subjected to oxygen and glucose deprivation, AMPK is activated, and pre-treatment with AICAR or CA-AMPK viral infection prolongs EC survival while inhibition exacerbates rates of apoptosis. The mechanism underlying this involves induction of Bcl-2 and survivin, through activation of NF κ B, which in turn appears to rely on AMPK α 1 signalling[277]. Induction of HO-1 is also important in this context. HO-1 is induced by an Nrf2 interaction with the antioxidant response elements in the *HMOX-1* promoter. AMPK-mediated cytoprotection against TNF α and ceramide was reduced by inhibition of HO-1[217].

Finally, AMPK activation in EC is also noted to have an anti-inflammatory action. Notwithstanding Liu *et al*'s study suggesting that NF κ B mediates AMPK's anti-apoptotic actions in EC, other work

suggests that AMPK activation with AICAR or viral over-expression attenuates NF κ B activation in EC by TNF α [333], leading to suppression of VCAM-1 and E-selectin. This leads to reduced monocyte adhesion to the EC surface[334]. Reducing AMPK α 2 signalling, by contrast, increases NF κ B translocation and degradation of I κ B α [331]. In bovine aortic endothelial cells (BAEC), AICAR treatment did not affect I κ B degradation and nuclear translocation of the p65 subunit, while binding of NF κ B to a κ B consensus sequence oligonucleotide was reduced in AICAR-treated cells, suggesting that AMPK inhibition of NF κ B occurs at the level of interference with DNA binding[335].

1.6.5 Clinical conditions resulting from aberrant AMPK activation

AMP kinase disease in people is an inherited condition caused by mutations in the gene encoding the AMPK γ 2 subunit (*PRKAG2*), and was first described in 2001[336]. It is rare: eleven families are described in the literature[337] and analysis of 100 consecutive referrals to a dedicated heart muscle unit found only 1 additional case[338].

Clinically the condition is characterised by left ventricular hypertrophy, mimicking hypertrophic cardiomyopathy, and by electrophysiological abnormalities including accessory conduction pathways and atrio-ventricular block[336, 339]. Later work has established that skeletal muscle is also abnormal, with proximal myopathy seen on electromyography (EMG), and a small number of patients also develop epilepsy with generalised tonic-clonic seizures[338].

Both families in the original description and the subsequent cases identified harbour missense mutations leading to amino acid substitutions in evolutionarily conserved regions of the protein: the CBS domains responsible for binding AMP. Altering γ 2 structure in this way does not affect the ability of the γ 2 subunit to associate with α and β subunits and form the complete complex, but does appear to affect its sensitivity to AMP, such that sensing the AMP:ATP ratio and energy state of the cell is impaired[340]. Murine models of the disease have been created, and have established that deposition of amylopectin, a glycogen-like polysaccharide, in myocytes is fundamental to disease development. It has been proposed that glycogen accumulation may lead to AMPK inactivation, and that this is responsible for the disease phenotype[341]. Amylopectin accumulation is also seen in myocardial sections from patients with the disease. Histological examination of quadriceps biopsies in those patients who also had a proximal myopathy demonstrate ragged red fibres with increased numbers of mitochondria. A mild excess of glycogen deposition was described in a subset, occasionally seen within mitochondria[338].

Single nucleotide polymorphisms (SNPs) in the gene encoding the AMPK α 1 subunit (*PRKAA1*) have been associated with an increased risk of developing gastric cancer in Chinese patients and with prognosis in colorectal cancer, but no association was seen with breast cancer [342-344]. The functional relevance of these SNPs is as yet unclear. Single nucleotide polymorphisms in *PRKAA1* have also been studied in the context of risk of coronary artery disease in patients with type II diabetes

in China, and an association seen between haplotype and protection from CAD but with increased CAD if the individual was also a smoker[345].

1.7 Methotrexate

1.7.1 Methotrexate in clinical practice

Methotrexate (MTX), an anti-metabolite drug that interferes with folic acid metabolism, is a well-established and effective anti-inflammatory agent central to the current treatment paradigms for RA[346, 347]. It is also used as a disease-modifying drug in other forms of inflammatory arthritis such as psoriatic arthritis and juvenile idiopathic arthritis; and as a steroid-sparing drug in many other chronic inflammatory conditions, including primary systemic vasculitis, sarcoidosis and idiopathic inflammatory myositis.

MTX was initially developed as a cancer-chemotherapy agent over 65 years ago. Following the discovery of folic acid and its role in cellular metabolism in 1946, effort went into developing anti-metabolites that would block the folic acid cycle, and aminopterin, a methotrexate precursor that potentially blocked folic acid metabolism, was discovered shortly after to be effective in inducing short remissions in acute leukaemia in children[348]. Aminopterin was first reported to be successful in treating RA by Gubner *et al* in 1951, who described 6 cases, 5 of whom improved[349]. However, this observation was not expanded upon until Hoffmeister reported success in treating 78 patients with intramuscular methotrexate in 1983[350]. Four randomised controlled trials were then conducted in the 1980s[351-354], which led to the widespread adoption of MTX as a treatment for RA.

MTX is now used as the first-line disease-modifying anti-rheumatic drug (DMARD) in RA treatment. DMARD denotes those agents that, in addition to improving the clinical features of the disease such as joint pain, swelling and stiffness, prevent progression of the disease, most clearly seen in the development of joint erosions on X-ray that signify permanent joint damage. The DMARDs include sulphasalazine and leflunomide, and are distinguished from NSAIDs and glucocorticoid steroids, that provide powerful symptomatic relief but do not prevent disease progression. MTX is the central DMARD because it is thought to have the best efficacy-to-toxicity ratio, when taken and monitored correctly[355]. A meta-analysis on the original 4 trials of MTX for RA found that patients treated with MTX had 26% fewer inflamed joints and 39% greater improvement in pain than those treated with placebo. Adverse effects resulting in discontinuation occurred in about one third of patients, but the vast majority of these were mild and less than 1% suffered serious adverse events[356]. These findings have led to a view of MTX as the benchmark treatment for RA against which all others, including biological agents, are measured.

MTX can be taken orally or parenterally by subcutaneous or intra-muscular injection, and the doses used in modern rheumatology practice (typically 10 - 25mg per week) are several orders of magnitude smaller than those when used as a cancer chemotherapy agent. Absorption is not significantly different with any route, but higher doses may be tolerated better if taken parenterally. Once in the circulation, approximately 50% is protein-bound. Side-effects include oral ulceration, stomatitis and

nausea commonly, which can be mitigated by the co-prescription of folic acid. More serious but rare effects include bone-marrow suppression with subsequent cytopenias, transaminitis progressing to cirrhosis if liver function abnormalities are disregarded, and pneumonitis. These can largely be avoided with appropriate prescription and monitoring blood tests.

1.7.2 Methotrexate and cardiovascular disease

There was initial concern that MTX would increase rates of CVD in RA, as its use is associated with increased plasma homocysteine concentrations[357]. Hyperhomocysteinaemia is associated with increased rates of CVD[358] and has toxic effects on endothelium including reduced NO availability and increased ROS generation[359]. However, the prescription of folic acid reduces homocysteinaemia, as it is required for the methylation of homocysteine into methionine[360], and further work has since established that MTX use is associated with a reduction in CVD in RA.

Although short case series and observational retrospective studies suggest that several DMARDs, including leflunomide and sulphasalazine, may reduce CVD, MTX is the drug for which the evidence is most secure[82]. Choi *et al*, in a retrospective study of over 1000 patients, found a 70% reduction in CVD mortality in RA patients taking MTX compared to other drugs; and more recent systematic literature reviews and a meta-analysis have confirmed this [79, 361, 362]. Moreover, MTX is also associated with improvement in functional markers of endothelial dysfunction[363]. Finally, MTX improved atherogenesis in the cholesterol-fed rabbit model. In this study, animals were treated with MTX 4mg/kg weekly by intravenous injection for 4 weeks, and a 75% reduction in atheroma area was reported, along with improvements in intima-media ratio and macrophage infiltration[364]. These findings have led to initiation of a trial of its use as a treatment to reduce atherosclerosis even in patients with no known inflammatory disease[15].

1.7.3 Mechanism of action of methotrexate

Despite its long history and widespread use the anti-inflammatory mechanism of action of MTX remains a subject of debate. Originally it was thought that antagonism of folate metabolism, which is the basis of its cancer killing actions, was also responsible for its anti-inflammatory effects. Other theories include accumulation and extracellular release of adenosine and increased intracellular oxidative stress.

Inhibition of folate metabolism

Inhibition of purine and pyrimidine synthesis is the established mechanism by which MTX kills malignant cells. Folates are co-enzymes derived from the diet and are essential for nucleotide synthesis, particularly purines and adenosine. Folate from the digestive tract is converted to dihydrofolate in the liver, and this enters cells via a folate transporter and is then converted intracellularly to tetrahydrofolate (TH4), the biologically active form, by dihydrofolate reductase

(DHFR). TH4 and its downstream metabolites are cofactors in many enzymatic processes, particularly those involved in purine synthesis. In addition, methyltetrahydrofolate promotes the conversion of homocysteine to methionine.

MTX is an analogue of folate and can therefore enter cells in the same way, via the folate transporter. It has a higher affinity for DHFR than does TH4, and hence inhibits TH4 production. In turn this prevents purine nucleotide synthesis and so stops cell proliferation, and in rapidly dividing cells induces apoptosis. However, MTX can also bind to and inhibit a number of other genes in folate and purine metabolism, including thymidylate synthase, which is the enzyme it has most affinity for (this is inhibited at MTX concentrations of 1nM, while inhibition of purine synthesis requires at least 10nM). Enzyme inhibition results in a cascade of effects, with build-up of metabolites leading to biological actions of their own. For example, MTX inhibition of DHFR, reducing generation of TH4, then results in reduced methylTH4, and thus reduced promotion of the conversion to methionine of homocysteine, hence raised levels of homocysteine.

As purine synthesis is essential for cellular proliferation, it seems reasonable that MTX, by reducing TH4 availability, could reduce disease activity in RA by inhibiting proliferation of activated T lymphocytes and macrophages in synovial tissue and elsewhere. Adverse effects such as bone marrow suppression and stomatitis are also likely to be secondary to the anti-proliferative effects of reducing folate metabolism. To support this, MTX induced a cell cycle block in *ex vivo* lymphocyte cultures derived from RA patients[365], and stimulated apoptosis of antigen-responsive T lymphocytes in culture. Furthermore, folic acid supplementation is associated with a reduction in the ability of MTX to lower titres of rheumatoid factor[366]. A recent study has shown that many folate cycle genes are upregulated in whole blood samples from RA patients, and their expression levels are normalised by taking MTX[367].

There are several reasons to suspect that inhibiting TH4 formation is not responsible for the anti-inflammatory action of MTX. Firstly, instead of high dose pulsed therapy as a cancer treatment, MTX for rheumatic disease is effective at low doses, (1-5g vs 10-25mg), taken on a weekly basis for many years. Secondly, the onset of anti-inflammatory action is slow, occurring over 4-6 weeks, whereas the half-life of MTX in the serum is 6-8h and is undetectable after 24h. Finally, supplementation with folic acid, routinely co-prescribed with MTX to prevent adverse effects, does not appear to alter its anti-inflammatory efficacy, although this has not been supported by all studies on the subject[368, 369]. Therefore, other mechanisms need to be invoked.

Adenosine accumulation and extra-cellular release

The relatively long duration of onset and offset of MTX's anti-inflammatory action was one of the factors that suggested folate inhibition was not its sole property. Intracellularly, MTX is converted to polyglutamate metabolites which persist for many weeks, and which inhibit a broader array of enzymes involved in nucleotide metabolism than does MTX itself, and the timing of its effects might be consistent with some of its biological action pertaining to these. The concentration of MTX polyglutamates in erythrocytes roughly correlates with therapeutic efficacy. MTX polyglutamates inhibit DHFR at least as well as MTX itself, but they inhibit AICAR transformylase more strongly[370, 371]. AICAR transformylase catalyses the conversion of the nucleotide AICAR into formyl-AICAR, a step in the production of hypoxanthine, and its inhibition leads to intracellular accumulation of AICAR. AICAR inhibits AMP deaminase, and can itself be metabolised to AICA-riboside (ZMP), which inhibits adenosine deaminase. This therefore leads to a rise in AMP and adenosine levels (Figure 1.5). As adenosine has potent anti-inflammatory actions[372], rises in adenosine have been posited as the anti-inflammatory mechanism of action of MTX[373].

There is good evidence that long term MTX treatment leads to AICAR accumulation. Patients taking low dose MTX to treat psoriasis excrete increased amounts of AICAR in their urine[374], and Cronstein *et al* reported increased AICAR concentrations in HUVEC and fibroblasts treated with MTX *in vitro*[375], and in splenocytes from MTX-treated mice *in vivo*[376]. Importantly, in both papers the MTX concentrations used were low, and administered over relatively long periods of time: 100nM over 48h in the case of *in vitro* work and 0.5mg/kg per week in the *in vivo* studies. There is also convincing evidence to suggest that MTX increases adenosine levels, and that this is sufficient to mediate anti-inflammatory effects. In the same paper, Cronstein *et al* showed that adenosine as a proportion of total nucleotides was enriched in HUVEC and fibroblasts treated with MTX, and that the adenosine release inhibited neutrophil adhesion to EC monolayers[375]. Moreover, *in vivo* studies of MTX 0.5mg/kg per week in mice using a carageenan air pouch model of inflammation showed increased adenosine concentrations in inflammatory exudates, accompanied by reduced lymphocyte accumulation. This was reversed by the addition of adenosine A2A receptor antagonists[376]. These effects were replicated in a model of inflammation in the rat gastro-intestinal tract, where MTX administration was associated with rises in adenosine and reduced leucocyte trafficking, and also in a model of rat adjuvant arthritis[377, 378]. Finally, drinking coffee appears to reduce the therapeutic efficacy of MTX in RA; which could be due to caffeine acting as an adenosine receptor antagonist[379].

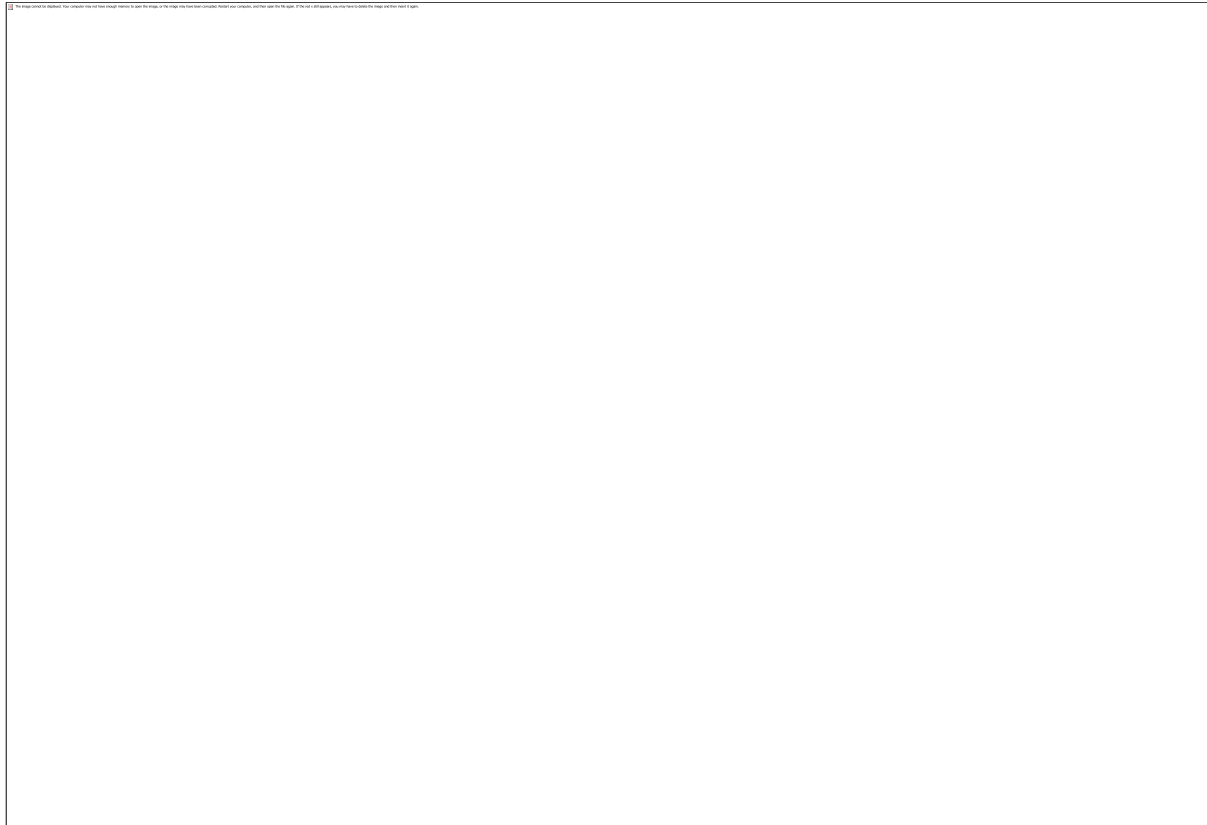


Figure 1.5 Mechanism of action of methotrexate. Adapted from Chan and Cronstein[369].

Generation of ROS

In vitro studies of the effects of MTX have shown that it reduces intracellular glutathione and increases cytosolic peroxide concentrations in monocytes, and this leads to inhibition of monocyte adherence to LPS-activated HUVEC[380]. However, the relevance of this to the patient is not clear, since intracellular glutathione levels in the inflamed synovium of the rheumatoid joint are already reduced well below that achieved by MTX *in vitro*, with high levels of ROS mediating injury in joint tissue in untreated patients. Moreover MTX has been shown to suppress IL-6- driven ROS production in synovial tissue[381].

1.7.4 Effects of methotrexate on vascular endothelium

The effects of MTX on vascular endothelium have not been extensively studied. The observations made so far generally fall into three groups: effects on EC proliferation and, by extension, angiogenesis; effects on homocysteine and NO production that might be thought pro-atherogenic; and effects on adenosine release and cell adhesion molecule expression that might be thought anti-atherogenic.

MTX prevents EC proliferation in culture at concentrations achievable, at least in the short term, in patient serum[382]. It does not however appear to stimulate EC apoptosis[383], although it does induce apoptosis in many cancer cell lines, activated T cells and keratinocytes[380, 384]. This

translates into inhibition of angiogenesis, which was observed in a rabbit corneal neovascularisation model when animals were treated with MTX 0.3 - 0.7mg/kg weekly for 8 weeks[385]. Inhibition of angiogenesis is probably a desirable effect in RA, as one would expect this to help with limiting synovial proliferation and may contribute to its disease-modifying properties. There is little known about whether MTX alters numbers of circulating endothelial progenitor cells, but a recent study of angiogenic T cells in RA suggests that MTX increases their numbers[386].

Through inhibition of DHFR, MTX might be expected to exert pro-atherogenic effects on EC. Firstly, inhibition of DHFR can lead to rises in homocysteine levels, although prescription of folic acid in addition to MTX probably prevents this causing significant problems in clinical practice. However, dihydrofolate reductase is also required as a salvage enzyme to maintain levels of tetrahydrobiopterin (BH₄), an essential co-factor for eNOS. Without BH₄, eNOS becomes uncoupled and produces superoxide instead of NO. Folic acid supplementation not only improves endothelial function even in patients without hyperhomocysteinaemia[387], it has been shown *in vitro* to upregulate EC DHFR expression and NO production, albeit using supra-physiological concentrations. Moreover, this is abolished if cells are pretreated with MTX 1μM. A small reduction in NO production was also noted when EC were treated with MTX alone[388].

Evidence that MTX might exert anti-atherogenic effects on EC comes from Cronstein *et al.* They showed that EC treated with MTX 100nM for 48h released increased adenosine, as a proportion of total nucleotides released. This treatment also inhibited neutrophil adherence[375]. Adenosine has anti-inflammatory, anti-atherogenic properties. Further anti-inflammatory effects of MTX on EC *in vitro* were demonstrated by Yamasaki *et al.*, who showed that MTX 1μM inhibits TNFα-induced ICAM-1 and VCAM-1 expression in HUVEC[382]. MTX has also been shown to reduce the TNFα-stimulated induction of the pro-inflammatory genes TNFα, IL-1β, VAP-1, CXCL-2 and TLR-2, and to up-regulate TGFβ in HUVEC, although the study showing this used very high concentrations of MTX over a short time period, and thus may not accurately reflect the situation *in vivo*[364].

Further anti-atherogenic effects of MTX have been demonstrated through its effects on reverse cholesterol transport in macrophages. Macrophages must be able to metabolise efficiently and process engulfed cholesterol: transformation to foam cells occurs when these processes are overwhelmed by too much lipid accumulation, as occurs in atherogenesis at the fatty streak stage. Cholesterol efflux in cells is regulated by two pathways: ATP-binding cassette transporter A1 (ABCA1) and cholesterol 27-hydroxylase[389]. In human THP-1 monocyte/ macrophages stimulated with IFNγ or COX-2 inhibitors, both of which down-regulated ABCA-1 expression, treatment with MTX restored levels, and also prevented foam cell formation. These results appeared to involve MTX's effects on adenosine levels, as inhibiting adenosine A_{2A} receptors abrogated them[390].

2 HYPOTHESIS

Mitigation of premature cardiovascular disease driven by chronic inflammation is a key goal in improving the outcome of patients with systemic inflammatory disease. Improved disease treatment and identification and modification of traditional risk factors will be important approaches, but a clear idea of which drugs offer most benefit to the vasculature would help enormously. To determine this, it is important to understand firstly how intrinsic mechanisms of endothelial protection operate, as these act to reduce endothelial dysfunction, the initial and reversible step in atherogenesis. Secondly, investigating the abilities of drugs used commonly in the treatment of chronic inflammatory disease to affect endothelial cytoprotective pathways should allow clinicians to offer their patients a more refined strategy to reduce their chances of developing atherosclerosis in the future.

Two key factors in preventing endothelial dysfunction physiologically are chronic exposure to pulsatile high flow LSS, and VEGF. Parts of the vasculature where blood flow has such characteristics rarely develop atherosclerotic plaques, while VEGF is a powerful driver of NO production. Both have been shown to affect similar cytoprotective pathways in EC, such as HO-1 induction, eNOS activation, and AMPK signalling. This led to the hypothesis that in combination VEGF and LSS induce synergistic improvements in endothelial cytoprotection.

Although MTX, the central DMARD used for long-term control of RA, reduces disease-associated CVD, little is known about the mechanisms that explain this effect. The anti-inflammatory effects of long-term low dose MTX are probably mediated by long-lasting polyglutamate metabolites of MTX, and these inhibit most powerfully AICAR transformylase, an enzyme involved in purine metabolism. The upstream effects of this inhibition are increased concentrations of AICAR, ZMP and AMP, and these are activators of AMPK. This led to the second hypothesis: that MTX activates AMPK in EC, improving resistance to inflammatory injury that ultimately protecting against atherogenesis.

To address these hypotheses, four specific questions were formulated:

Firstly, does exposure of EC, conditioned by LSS, to VEGF result in a synergistic induction of cytoprotective enzymes such as HO-1? If so, can the underlying signalling pathways be mapped out?

Secondly, does long-term low dose MTX activate cytoprotective pathways in EC, particularly AMPK, and does this result in functional improvements in EC survival?

Thirdly, how does activation of AMPK by MTX lead to changes in expression of cytoprotective effector proteins?

Finally, does MTX activate AMPK in the vasculature *in vivo*, and does it ameliorate the inflammatory vasculopathy that is a feature of the SLE-like illness in (NZWxBXSB)F1 male mice?

3 MATERIALS AND METHODS

3.1 Cell culture techniques and analysis

3.1.1 Isolation and culture of HUVEC

The experimental use of HUVEC was approved by the Hammersmith Hospital Research Ethics Committee (ref. no.06/QO46/21), and isolation and culture was carried out as described by Jaffe *et al*[391]. Umbilical cords were collected in Hanks' buffered saline solution (HBSS) containing calcium and magnesium (Sigma-Aldrich Ltd.), supplemented with 1mM sodium pyruvate (Gibco by Life Technologies) and 0.1mg/ml gentamicin (Gibco). A sterile three-way connector tap (Bunzl Healthcare) was inserted into the umbilical vein and flushed with warmed HBSS without Ca/Mg. A second three-way tap was inserted in the other end and the vein was filled with 0.5mg/ml collagenase in HBSS and incubated for 10 minutes at 37°C. The cord was massaged to detach the EC, flushed with warm HBSS and the collagenase solution was collected into a 50ml Falcon tube (Helena Biosciences). The solution was centrifuged at 1200rpm for 10 minutes, the supernatant was discarded, and the pellet resuspended in 5ml M199 EC culture medium (Sigma) supplemented with 100µg/ml penicillin and 100µg/ml streptomycin (Gibco), 2mM L-glutamine (Gibco), 20% fetal calf serum (FCS; Biosera), 30µg/ml endothelial cell growth factor (ECGF; Sigma) and 10 units/ml heparin. The EC were transferred to a 1% gelatine-coated T25 culture flask (Costar) and cultured for 24 hours before washing with HBSS to remove debris and blood, and the addition of 5ml of fresh HUVEC culture medium.

3.1.2 Culture of HMEC and HAEC

Third passage human microvascular endothelial cells (HMEC; gift from Dr E Ades, CDC, Atlanta) were retrieved from long term storage at -196°C in liquid nitrogen and cultured in MCDB-131 medium (Gibco) supplemented with 100µg/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 10 units/ml heparin, 15% FCS and 10µg/ml EGF (Peprotech).

Fifth passage human aortic endothelial cells (HAEC; Promocell) were retrieved from long term storage at -196°C in liquid nitrogen (with thanks to Dr D Calay) and cultured in M199 supplemented with 100µg/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 10 units/ml heparin, 20% FCS and 30µg/ml ECGF.

3.1.3 Passage of EC

EC were passaged once they formed a fully confluent monolayer (Figure 3.1), every 2-4 days. The exhausted medium was aspirated, the cells were washed in HBSS and then incubated for 2 minutes with 1ml trypsin/ ethylenediaminetetraacetic acid (EDTA; MP Biomedicals) to promote detachment from the gelatine basement membrane. They were resuspended in an appropriate volume of complete medium to allow passage into a T75 flask (if first passage) or a 1:2 or 3 split if later passages. HUVEC were used at passage 2-4; HMEC were used at passage 5 and above and HAEC were used at passage 7 and 8.

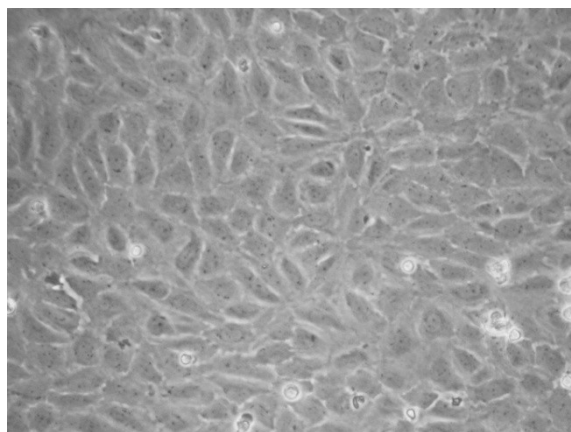


Figure 3.1 Photomicrograph of confluent passage 3 HUVEC in static culture (x200 magnification)

3.1.4 Stimulation of EC with pharmacological agents and growth factors

EC were harvested with trypsin as described in section 2.3 and seeded at 70% confluence into 12 or 6-well plates (1.75 or 3.5×10^5 cells per well) coated in 1% gelatine. Following overnight incubation in M199 containing 10% FCS and $15 \mu\text{g/ml}$ ECGF to allow the EC to settle, medium was aspirated, the cells were washed in HBSS and fresh medium was added. Experimental agents including growth factors and pharmacological agents were then added at concentrations described in the results chapters and in Table 2.1, along with appropriate negative vehicle controls (either double distilled water (ddH_2O) or dimethyl sulphoxide (DMSO)).

Agent	Company	Concentration
AICAR- 5' phosphate	Calbiochem	1mM
Compound C (AMPK inhibitor)	Calbiochem	5 μ M
Dimethyl sulfoxide	Sigma	
Folic acid	Sigma	50nM - 50 μ M
Hemin	Sigma	0.5 μ M
Hydrogen peroxide	Sigma	
Methotrexate	TOCRIS	0.1nM – 2 μ M
Mito-TEMPO	Enzo Life Sciences	5 μ M
N-acetyl cysteine	Enzo Life Sciences	10mM
Tumour necrosis factor- α	R&D Systems	0.1-10ng/ml
Vascular endothelial growth factor (VEGF A ₁₆₅)	Peprotech	25ng/ml

Table 3.1 Growth factors and pharmacological agents used to treat EC

3.1.5 RNA interference by lipid-mediated transfection

Transfection of EC with short interfering RNA (siRNA) was carried out by a liposomal method: complexing siRNA with geneFECTOR transfection agent (VennNova Inc), a proprietary lipid compound. 3x10⁵ HUVEC were plated onto gelatine-coated 6 well plates in M199/10% FCS/15 μ g/ml ECGF and allowed to settle overnight, aiming for 50% confluence. The following day they were washed with ice cold Optimem medium (Gibco) then fed with 800 μ l pre-warmed Optimem per well. A solution of geneFECTOR was prepared containing 6 μ l geneFECTOR and 100 μ l Optimem per well, and a solution of siRNA directed against the gene of interest and one of a scramble control siRNA (Dharmacon) was prepared, diluting 2.5 μ l of 20 μ M stock in 100 μ l Optimem per well. For each siRNA reaction 100 μ l siRNA solution was mixed with 100 μ l geneFECTOR solution and incubated at room temperature for 5 minutes. The entire 200 μ l siRNA-geneFECTOR solution was then added dropwise to each well and incubated at 37°C for 6h. The Optimem/ siRNA/ geneFECTOR solution was then aspirated and cells allowed to recover in EGM-2 medium (Lonza) overnight. The following day, this was aspirated and EC were treated with MTX or DMSO in M199/ 10%FCS/ 15 μ g/ml ECGF for 48h before lysis.

Target Gene	siRNA name	mRNA Target Sequence 5' – 3'	Company
AMPK α_1	Hs_PRKAA1_5	CCC ACG ATA TTC TGT ACA CAA	Qiagen
AMPK α_2	Hs_PRKAA2_6	CCG AAG TCA GAG CAA ACC GTA	Qiagen
CREB-1	CREB-1 pooled sequence siGENOME SMARTpool	GAG AGA GGT CCG TCT AAT G CGT ACA AAC ATA CCA GAT T GAG TGG AGA TGC AGC TGT A TGA CTT ATC TTC TGA TGC A	Dharmacon

Table 3.2 siRNA sequences

3.1.6 Exposure of EC to LSS

The effects of exposure to LSS on EC were investigated in the Cytodyne streamer flow system (Cytodyne). This consists of a parallel plate flow chamber and a recirculating flow loop as shown in Figure 3.2, with an upper and lower reservoir. EC seeded onto a glass slide and are mounted into a medium-filled chamber and held in place by vacuum suction. The chamber is then connected to a circuit of medium with two reservoirs. Medium is pumped from the bottom reservoir to the top by a peristaltic pump (VL500 Basic, Verderflex). It then flows down over the EC mounted within the parallel plate chamber, and back into the bottom reservoir. The height of the top reservoir determines the degree of shear stress, which was previously measured by Dr A Kinderlerer and determined to be 12-20 dynes/cm², similar to that in the human aorta.

HUVEC were seeded onto 75 x 38mm glass slides (Fisher Scientific) coated in 1ml 10 μ g/ml fibronectin diluted in HBSS and incubated in complete medium in 90mm Petri dishes (Nunc) for at least 16 hours to allow establishment of confluence. They were then mounted into the Cytodyne streamer flow system or left in static conditions in the Petri dish.

In experiments, two circuits were set up simultaneously and were placed in a chamber heated to 37°C, connected to a continuous supply of air and 5% CO₂ and left to run for 16 to 24h to allow equilibration of EC to flow. Drugs and growth factors were then added to one circuit for 4 to 24h before cell lysis. This was achieved by rapid disconnection of the glass slide from the parallel plate chamber and immediate immersion in ice cold HBSS, before washing and lysis for RNA or protein extraction.

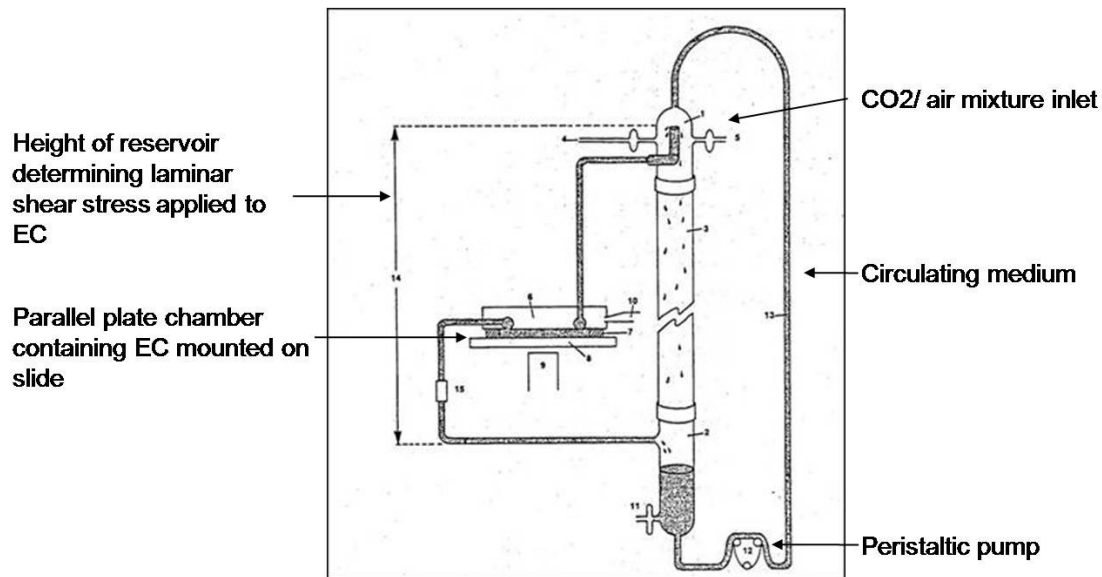


Figure 3.2 Cytodyne streamer flow system.

Characterisation of response and optimisation of housekeeping genes

The original experiments to validate the Cytodyne streamer flow system in our department were carried out previously by Dr A Kinderlerer and Dr F Ali [189, 248]. Initial characterisation and optimisation experiments by me demonstrated that after 24h exposure to LSS, HUVEC changed their morphology and realigned in the direction of flow (Figure 3.3).

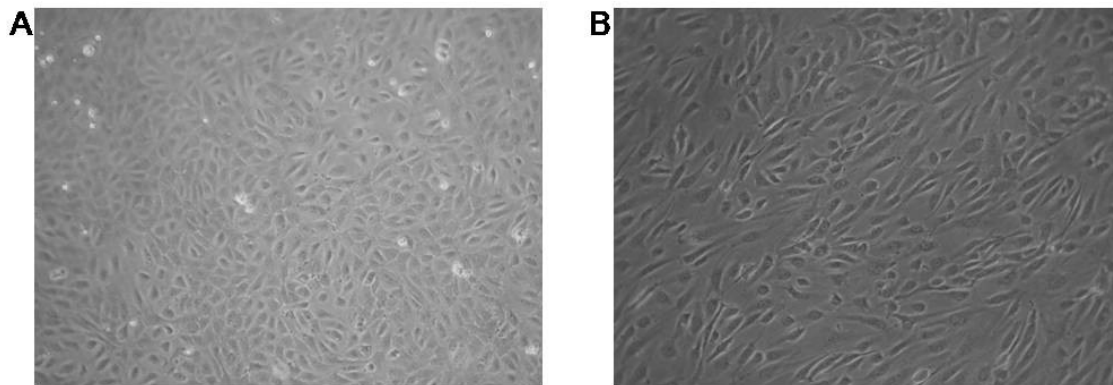


Figure 3.3 Photomicrographs of HUVEC exposed to static or laminar shear stress conditions. A: static culture; B: LSS 20 dynes/cm² for 24h; magnification x100.

For mRNA analysis by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) of HUVEC exposed to LSS or static conditions, three housekeeping genes were used: β -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). It became apparent from studying cycle threshold (cT) values after several experiments that β -actin mRNA levels were consistently downregulated when HUVEC

were exposed to LSS for 24h or more, while GAPDH and HPRT were unchanged (Figure 3.4). mRNA results were compared primarily to GAPDH thereafter.

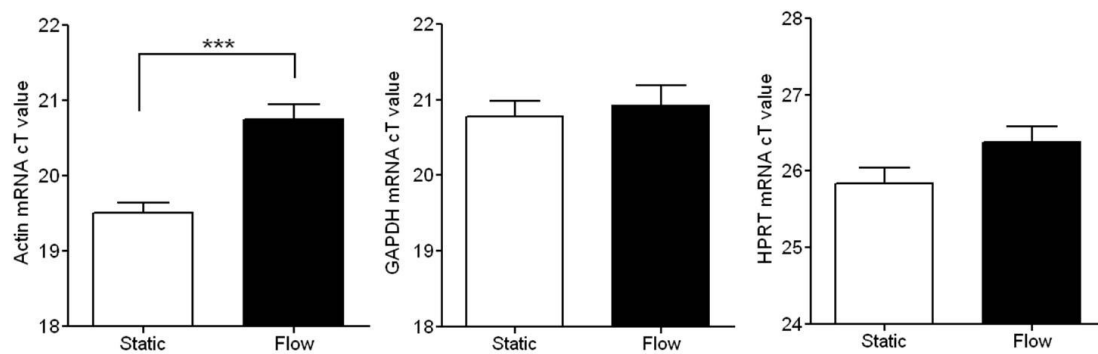


Figure 3.4 cT values for housekeeping genes under static or LSS conditions.

3.1.7 Cell number assay

This was carried out using the CellTiter 96 AQueous One Solution cell proliferation assay (Promega). HUVEC were plated in a 1% gelatine coated flat bottomed 96 well plate at 5000 cells/well and treated in quadruplicate with MTX, DMSO control or serum starvation for 48h (in M199 with 0.1% BSA). 1.9mg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was mixed with 300μM phenazine ethosulfate (PES) and 20μl was added to 100μl medium per well. HUVEC were incubated in the MTS/PES medium for 2h and the colour change was then quantified by measuring absorbance at 490nm on a plate reader.

3.1.8 Flow-cytometric analysis by FACS

HUVEC grown under experimental conditions in 6 well plates were detached with 200μl trypsin/EDTA and split equally into two fluorescence-activated cell sorter (FACS) tubes (Marathon) with 1-2ml FACS medium (HBSS supplemented with 1% FCS) added. After centrifugation at 1800rpm for 5 minutes, the cells were incubated with the primary antibody or FACS medium (as negative control) at 4°C for 30 minutes. 2ml FACS medium was then added and the cells centrifuged again before incubation with a species-specific FITC-labelled secondary antibody at 4°C for a further 30 minutes. After further centrifugation in FACS medium, the cell pellet was resuspended in cold phosphate-buffered saline (PBS) and kept on ice. The antibodies used are shown in Table 3.2.

Samples were analysed using a Beckman Coulter CyAn flow cytometer. A gate was drawn around the EC using the forward scatter/ side scatter histogram and 10 000 cells within this gate were counted and fluorescence analysed (as mean fluorescence intensity (MFI)). Relative fluorescence intensity (RFI) was calculated by dividing the MFI of cells stained with antibody of interest by that of the

unstained negative control. The data was processed using the associated Summit 4.3 software. Representative histograms and plots are shown in Figure 3.5.

Target Antigen	Source Species	mAb Name	Supplier	Concentration
FITC-labelled polyclonal anti-mouse IgG	Rabbit		DAKO	1:50
CD31	Mouse	P2B1 α	Developmental Studies Hybridoma Bank, USA	Supernatant
Endoglin	Mouse	RMAC8	Dr d'Apice, St Vincent's Hospital, Australia	Supernatant
ICAM-2	Mouse	CBR-IC/2	Dr Springer, Harvard University, USA	Supernatant
DAF	Mouse	1H4	Departmental	Supernatant
ICAM-1	Mouse	6.5B5	Departmental	Supernatant
VCAM-1	Mouse	1.4C3	Departmental	Supernatant

Table 3.3 Antibodies used in FACS analysis. All are monoclonal unless stated otherwise.

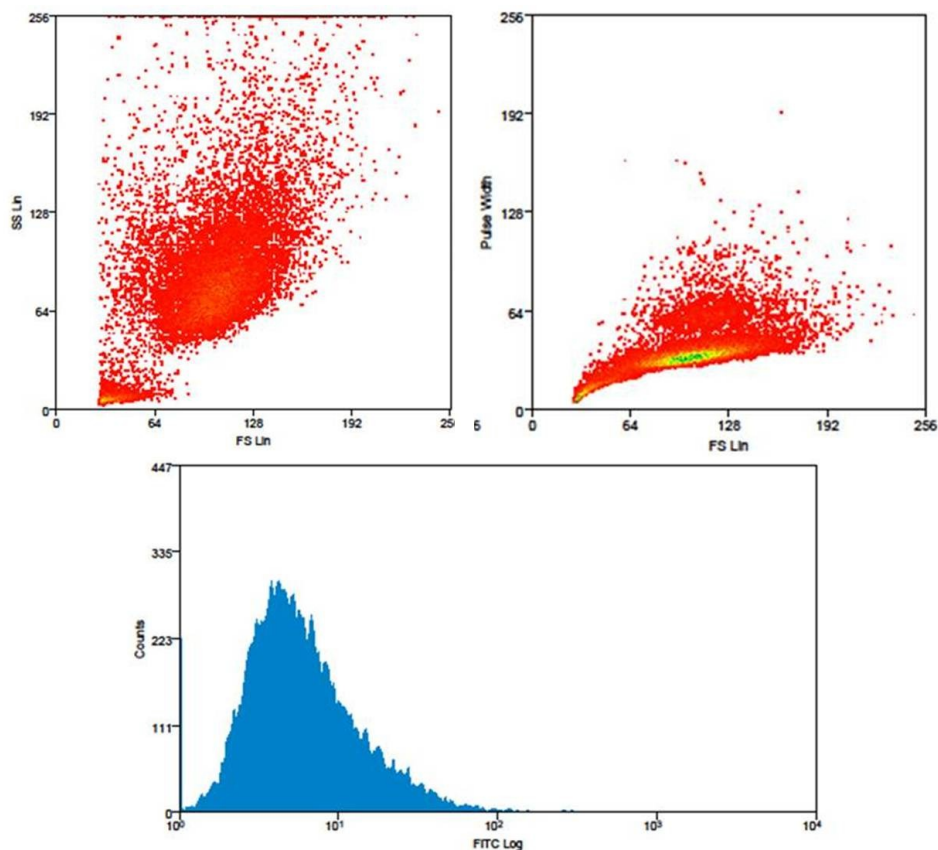


Figure 3.5 FACS analysis of HUVEC. SS Lin: side scatter linear scale; FS Lin: forward scatter linear scale; FITC Log: fluorescein isothiocyanate staining log scale.

3.1.9 Cell cycle analysis by propidium iodide staining

Cell cycle analysis was carried out by staining fixed EC with propidium iodide (PI), a fluorescent dye that binds nucleic acids by intercalation between base pairs. If RNA is removed, the DNA content of cells can be quantified and thus the cell cycle stage determined.

HUVEC pre-treated with MTX, DMSO control or serum starvation in M199/0.1% BSA were harvested with 200µl trypsin/EDTA, centrifuged and resuspended in 200µl PBS. They were fixed by adding the cell suspension dropwise into vortexing 70% ethanol/PBS, then incubating at 4°C for 30 minutes. After centrifugation at 2000rpm for 15 minutes, the pellet was resuspended in 150µl PBS and transferred to FACS tubes containing 500µl PI staining solution (50µg/ml PI) (Sigma), 0.1% Triton X-100 (Sigma), 18mg/ml EDTA (Sigma), 20u/ml RNase (Sigma), PBS). They were then incubated in darkness at 37°C for 45 minutes before reading by FACS, counting 10000 cells.

3.1.10 Detection of apoptosis

Apoptosis was induced in HUVEC by glucose deprivation for 18h. Glucose deprivation was achieved by incubating cells in sterile-filtered modified HBSS without glucose (see appendix for recipe), supplemented with L-glutamine, penicillin/streptomycin and 2% BSA.

Apoptosis detection was carried out using Annexin V and PI staining. Cells were washed, trypsinised, resuspended in FACS medium, divided into two samples per condition and centrifuged at 1200rpm. The resulting pellets were incubated with a perCP-conjugated anti-phosphatidylserine (Annexin V) antibody (BD Pharmingen) diluted 1:20 in Annexin V binding buffer (BD Pharmingen) or in buffer alone for 30 minutes at 4°C. Samples were analysed by FACS, with unstained samples read first and then briefly incubated with 0.5mg/ml PI (Sigma) mixed 50:50 with 2x PBS, before Annexin V staining and PI positivity was measured.

Trypan blue staining was also used as an alternative method of measuring cell death. Briefly, cells seeded into a 24 well plate in which apoptosis had been induced were trypsinised and suspended in 100µl of a 50:50 mix of 0.4% trypan blue staining solution (Sigma) and FACS medium. Numbers of living and dead cells (which were stained blue having become permeable to trypan blue) were counted in 5 squares of a haemocytometer.

3.1.11 Detection of intracellular ROS production

HUVEC were treated with MTX or DMSO for 48h. 5'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was thawed, dissolved in DMSO to create a stock concentration of 1µg/ml and loaded at 5µM in pre-warmed PBS to each well followed by incubation in darkness at 37°C for 45 minutes to allow its take-up by the cells. The cells were allowed to recover in medium for 15 minutes before further incubation with H₂O₂ for 1h. They were then trypsinised, resuspended in PBS and the fluorescent signal was read by counting 10000 cells by FACS.

3.2 Molecular biology techniques

3.2.1 Western blotting

Cell lysis for protein analysis

EC exposed to experimental conditions were lysed at 4°C for 20 min in RIPA buffer (Thermo Scientific) supplemented with complete protease inhibitor cocktail (Roche) and phosphatase inhibitors (Phostop, Roche) and then scraped into 0.5ml tubes. Samples were sonicated for 1 min and centrifuged at 14 000 rpm for 20 min at 4°C in a microcentrifuge (Sigma). The supernatant was harvested and stored at -80°C.

Measurement of protein concentration

Lysate protein concentration (to ensure equal loading of western blot gels) was measured using the DC protein assay (Bio-Rad). Lysates were loaded into a 96 well plate in triplicate, along with a BSA standard (Bio-Rad). Additional reagents were added as per the manufacturer's instructions to produce a colour intensity proportional to the sample protein concentration. Following incubation for 15 minutes, samples were read at 595nm on a plate reader (Synergy HT, Bio-Tek).

Electrophoresis, transfer, antibody probing and developing

Loading samples were composed of 10 or 20µg sample protein, 7.5µl NuPage sodium dodecyl sulphate (SDS) 4X sample buffer (Invitrogen), 2µl 1M dithiothreitol (DTT) (Fluka) and ddH₂O up to a total volume of 30µl. They were boiled for 5 minutes, then loaded into 10% or 4-12%, 1 or 1.5mm, 15 or 10 well NuPage gels (Invitrogen), with at least one lane loaded with 10µl See-Blue molecular weight marker (Invitrogen). Gels were run in a Novex minicell tank (Invitrogen) in NuPage MOPS SDS running buffer (Invitrogen), at 150V for 90 minutes.

Transfer to a PVDF membrane was performed using semi-dry blotting apparatus (Bio-Rad), at 0.1A per gel for one hour. Membranes were blocked in 5% BSA/ tris-buffered saline-tween (TBS-T) for one hour and incubated overnight with the primary antibodies, diluted in TBS-T, on a rocker at 4°C. The next day, membranes were washed in TBS-T and incubated at room temperature for one hour with a species-matched horseradish peroxidase (HRP)-labelled secondary antibody, diluted in 5% BSA/TBS-T. After another wash in TBS-T, membranes were developed using ECL or ECL-plus HRP detection systems (GE Healthcare) and exposure to Biomax light film (Kodak) in a Compact X4 developer (Xograph Imaging Systems). The primary and secondary antibodies used are shown in Table 3.4.

Target Antigen	Species	Supplier	Concentration
AMPK α	Rabbit	Cell Signaling	1:1000
Phospho AMPK α ^{Thr172}	Rabbit polyclonal	Abcam	1:2500
CREB	Rabbit	Cell Signaling	1:500
Phospho CREB ^{Ser133}	Rabbit	Cell Signaling	1:1000
HO-1 (OSA111)	Mouse monoclonal	Abcam	1:2000
MnSOD	Rabbit monoclonal	Assay Designs	1:2500
GAPDH	Mouse monoclonal	Millipore	1:25000
α -Tubulin	Mouse monoclonal	Sigma	1:25000
Normal IgG ₁	Mouse	DAKO	100mg/L
HRP-tagged anti-mouse IgG	Goat	DAKO	1:1500
HRP-tagged anti-rabbit IgG	Swine	DAKO	1:3000

Table 3.4 Antibodies used in immunoblotting.

Densitometry

An HP solution centre scanner was used to scan films, and band pixel density was quantified using Image J software. Optical density of the band of interest was corrected for any loading inaccuracies by dividing by the optical density of the loading control band (α -tubulin or GAPDH). Results were expressed relative to the untreated control.

3.2.2 Phospho-kinase array

A human phospho-kinase array kit (Proteome Profiler antibody arrays, R&D systems) was used to investigate protein phosphorylation changes in EC exposed to LSS and VEGF. HUVEC pooled from 3 separate cords were mounted in the Cytodyne streamer system and exposed to LSS for 24h or kept in static conditions, before the addition of VEGF 25ng/ml or vehicle control for 15 minutes. This time point was chosen on the basis of a preliminary experiment indicating good phosphorylation of ERK1/2 by VEGF after 15 minutes. The HUVEC were washed with PBS, then lysed in the kit Lysis Buffer according to the manufacturer's instructions. The lysate was spun at 14000g for 5 minutes, and the supernatant collected and stored. A Bradford protein assay was done to ensure loading of equal quantites of protein across the four conditions.

To use the array, each membrane (2 sections, A and B, for each condition) was blocked for 1h at room temperature. The cell lysates were diluted in the Array Buffer such that the maximum quantity of protein was used while ensuring equal quantities in each condition, and the diluted cell lysate was added to each array membrane, and incubated overnight at 4°C on a rocking platform.

The following day, the membranes were washed three times in the kit Wash Buffer, and then incubated with the kit Detection Antibody Cocktail at room temperature for 2h. After a further wash, they were incubated with streptavidin-HRP for 30 minutes before exposure with ECL-plus chemi-luminescent reagents and detection with X-ray film. Representative pictures are shown in Figure 3.6. A transparent overlay was used to identify the individual phosphorylated proteins, and the signal density for each spot was quantified in Image J.

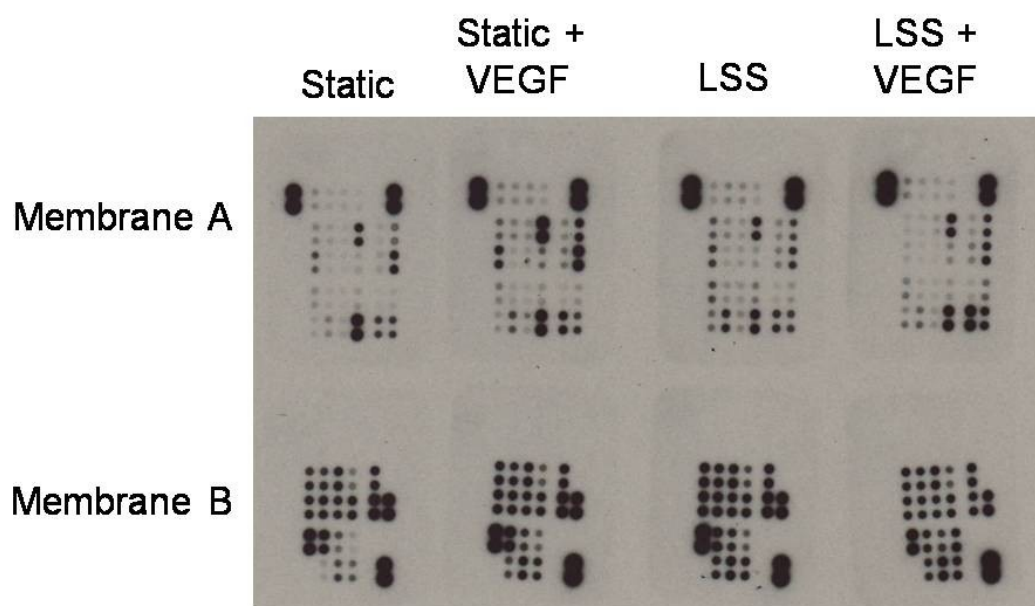


Figure 3.6 Phospho-kinase array image after exposure in ECL-plus for 30s. LSS: laminar shear stress.

3.2.3 Quantitative real-time polymerase chain reaction

RNA isolation

The RNeasy Mini Kit (Qiagen) was used to isolate RNA. EC were lysed in RLT buffer supplemented with 10µl/ml β-mercaptoethanol. To disrupt the cell contents, lysates were either transferred to a QiaShredder column and centrifuged at 15000g for 2 min (Sigma 1-13 centrifuge) or passed through a 21G needle with a 1ml syringe 10-15 times. Each sample was mixed with an equal volume of 70% ethanol, then transferred to an RNeasy column and centrifuged at 15000g for 15 seconds, trapping the nucleic acids in the column. 350µl RW1 buffer was added and the sample was again centrifuged. 10µl deoxyribonuclease (DNase), mixed with 70µl RDD buffer (Qiagen), was added to each tube and incubated for 15 minutes at room temperature. 350µl RW1 buffer was again added to the column, followed by centrifugation and two washes in 500µl RPE buffer. Finally, RNA was eluted by adding 30µl ddH₂O. The eluted RNA was quantified on an Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech Ltd), by comparing the absorbance at 260nm and 280nm of a 1:100 diluted sample of RNA (in ddH₂O) to a sample of ddH₂O alone.

Complementary DNA synthesis

For synthesis of complementary DNA (cDNA), RNA was diluted to 1µg/10µl ddH₂O in 0.2ml PCR tubes (Fisher Scientific). 1µl Oligo dT (Invitrogen) and 1µl 10mM dNTP (Roche Diagnostics) were added. Samples were heated at 65°C for 15 minutes using a thermocycler (T3 Thermocycler, Biometre, GmbH), then placed on ice and 4µl 5x first strand buffer (Invitrogen), 2µl 0.1M DTT (Invitrogen) and 1µl ddH₂O were added. The thermocycler was set to a hot start programme, and when 42°C was reached 1µl Superscript II RNase Reverse Transcriptase (Invitrogen) was added to each tube. Samples were then heated for 55 min at 42°C and 15 min at 70°C.

Quantitative real time PCR

qRT-PCR was performed on a Bio-Rad CFX96 Real Time System C1000 Thermal Cycler with β-actin, GAPDH and HPRT as housekeeper genes. Each sample was run in triplicate.

5µl cDNA diluted 1:25 in ddH₂O was pipetted into the wells of a 96-well reaction plate (Bio-Rad). Validated primers were diluted 1:10 to create a working solution. 1µl of each forward and reverse primer working solution was mixed with 12.5µl SYBRgreen jump start taq ready mix (Quanta Biosciences), and 6.5µl ddH₂O for each reaction. The plate was centrifuged at 335 x g for 1 minute and run on the thermal cycler using the protocol shown in Table 3.5.

Step	Number of cycles	Temperature ($^{\circ}\text{C}$)	Time (s)
1	1	95	180
2	40	95	10
3	40	56	45 Plate read
4 Melt Curve	1	56-95 in 0.5°C increments	10 per increment Plate read

Table 3.5 Bio-Rad thermal cycler protocol.

qRT-PCR works via the generation of a fluorescent signal which is measured after each cycle to detect the replication of the PCR product. The readout is given as the number of cycles required for the fluorescent signal to surpass the cycle threshold. The fewer the cycles required, the greater the number of mRNA transcripts present in the sample. Measurement of the signal from constitutively expressed genes allows normalisation of results between samples. Data was analysed using the comparative cycle threshold method ($\Delta\Delta\text{Ct}$). Representative qPCR curves are shown in Figure 3.7.

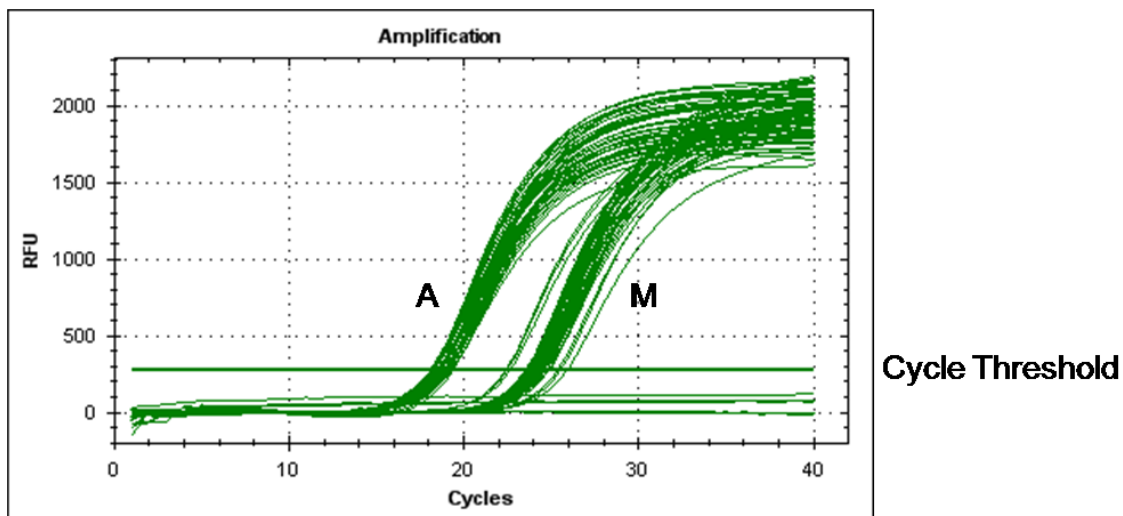


Figure 3.7 Representative qRT-PCR curves. A: β -actin; M: MnSOD.

Primer validation

Each primer pair was tested for the efficiency of its binding and a melt curve was run to check for the formation of primer dimer. 10-fold dilutions of cDNA were pipetted in duplicate, along with a no template control (ddH₂O), and the iCycler protocol was run. The resulting cT values were plotted for each pair and a best fit line drawn. Efficiency of 90% or more was considered adequate, with a slope gradient close to 1. Primer sequences are shown in Table 3.6. A representative melt curve for MnSOD and actin is shown in Figure 3.8.

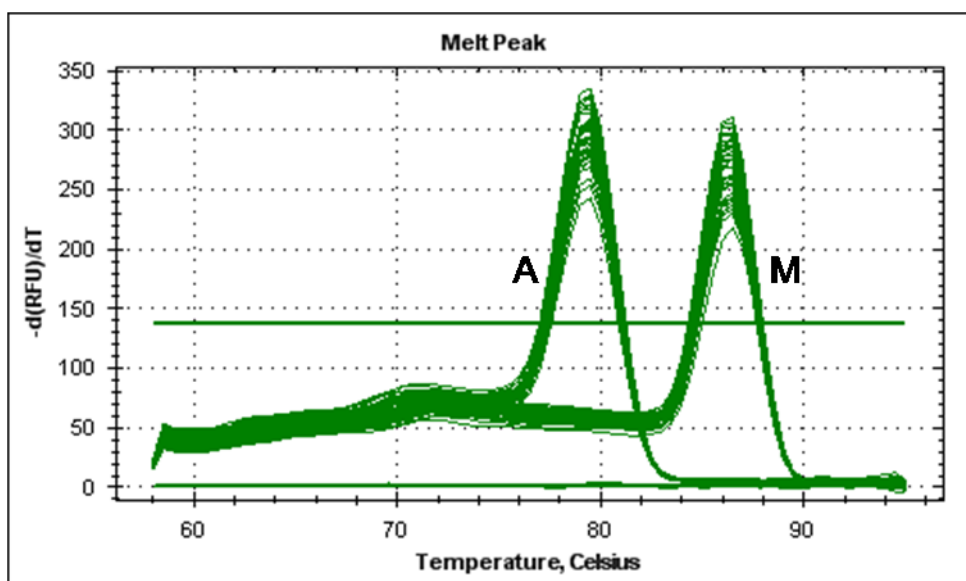


Figure 3.8 Representative qRT-PCR melt curves. A: β -actin; M: MnSOD.

mRNA Target	Sequences
A1	Forward 5'-CAC AGG AGA ATG GAT AAG GCA AA-3' Reverse 5'-AGT CAT CCA GCC AGA TTT AGG TTC-3'
AMPK α 1	Forward 5'-TCA GGA AGA TTG TAT GCA GGC CCA-3' Reverse 5'-TTC ATG GGA ATC CAC CTG CAG GAT TA-3'
AMPK α 2	Forward 5'-CGA GCT ATG AAG CAG CTG GA-3' Reverse 5'-GAA CGC TGA GGT GTT GAG GA-3'
β -Actin	Forward 5'-GAG CTA CGA GCT GCC TGA CG-3' Reverse 5'-GTA GTT TCG TGG ATG CCA CAG GAC T-3'
Bcl-2	Forward 5'-GCC CTG TGG ATG ACT GAG TA-3' Reverse 5'-GGC CGT ACA GTT CCA CAA AG-3'
DAF	Forward 5'-CCT TAA GGG CAG TCA ATG GT-3' Reverse 5'-CGG CAC TCA TAT TCC ACA AC-3'
eNOS	Forward 5'-CTG GCT GTC TCC ATG GAC CT-3' Reverse 5'-CCA CCA TGG TGA CTT TGG CT-3'
GAPDH	Forward 5'-CAA CAG CCT CAA GAT CAT C-3' Reverse 5'-GAG TCC TTC CAC GAT ACC-3'
HO-1	Forward 5'-CAG TCT TCG CCC CTG TCT AC-3' Reverse 5'-CTG GTG TGT AGG GGA TGA CC-3'
HPRT	Forward 5'-TTG GTC AGG CAG TAT AAT CC-3' Reverse 5'-GGG CAT ATC CTA CAA CAA C-3'
KLF-2	Forward 5'-GCA AGA CCT ACA CCA AGA GTT CG-3' Reverse 5'-CAT GTG CCG TTT CAT GTG C-3'
MnSOD	Forward 5'-AAG GGA GAT GTT ACA GCC CAC-3' Reverse 5'-GTC CAG AAA ATG CTA TGA TTG A-3'
NQO-1	Forward 5'-GGG CAA GTC CAT CCA ACT G-3' Reverse 5'-GCA AGT CAG GGA AGC CTG CA-3'
Trx	Forward 5'-GGT GTG CGC CTT GCA A-3' Reverse 5'-TCA AGG AAT ATC ACG TTC CAA TAC TT-3'
Trx reductase	Forward 5'-CCA CTG GTG AAA GAC CAC GTT-3' Reverse 5'-AGG AGA AAA GAT CAT CAC TGC TGA T-3'
UCP-2	Forward 5'-GGA TAC TGC TAA AGT CCG GT-3' Reverse 5'-CCA TTG TAG AGG CTT CGG G-3'
VEGFR-2	Forward 5'-AAT GAC ACT GGA GCC TAC AAG-3' Reverse 5'-CAC CGA GAC CTG GAA TCA C-3'

Table 3.6 qRT-PCR primer sequences.

3.2.4 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP), to investigate whether CREB binds the SOD2 promoter in EC treated with MTX, was carried out using the ChIP-IT Express kit (Active Motif).

Cell stimulation and fixation

HUVEC from 3 cords were pooled and plated in 15cm cell culture plates in order to give approximately 10^6 cells per condition, then treated with MTX 100nM for 48h or DMSO control. Cells were washed with ice-cold PBS then fixed in 1% formaldehyde in HBSS by rocking at room temperature for 5 minutes, followed by a glycine stop-fix solution to stop the fixation reaction. The cells were then scraped off using a solution of PBS with 500 μ M phenylmethylsulphonyl fluoride (PMSF) as a serine protease inhibitor, transferred to a 15ml conical tube on ice and pelleted by centrifugation. The supernatant was discarded, 1 μ l 100mM PMSF and 1 μ l protease inhibitor cocktail (PIC) were added and the pellets were frozen at -80°C.

Cell lysis and chromatin shearing by sonication

The cell pellets were resuspended in 1ml ice-cold Lysis Buffer supplemented with PMSF and PIC, then incubated on ice for 1h, before homogenisation in a pre-chilled dounce homogeniser with 20 strokes to improve nuclei release. Adequate cell lysis was checked by examining a 10 μ l sample in a haemocytometer. The nuclei were pelleted by centrifugation, the supernatant discarded and the pellets resuspended in shearing buffer. The chromatin was sheared at 4°C in a Diagenode Bioruptor for 4 minutes with 30s pulses of shearing followed by 30s rest in order to generate chromatin fragments with an average length of 500bp. The sheared chromatin was centrifuged and the supernatant aliquoted and stored at -80°C.

Ascertaining DNA concentration and shearing efficiency

The DNA concentration of each sample was checked to ensure an adequate chromatin yield from the fixation, lysis and shearing steps. A 50 μ l sample from each condition was mixed with 150 μ l ddH₂O and 10 μ l 5M NaCl, then heated at 65°C in a thermocycler overnight. The following day they were treated with RNase and proteinase K, and a phenol/chloroform extraction performed to purify the DNA. 200 μ l 1:1 phenol/chloroform TE saturated pH8 was added to each sample, vortexed and centrifuged at maximum speed for 5 minutes. The top aqueous layer was removed and added to 20 μ l 3M sodium acetate pH5.2 and 500 μ l 100% ethanol, vortexed and placed at -80°C for 1h. The sample was centrifuged twice at maximum speed, then the pellet left to air dry before resuspension in ddH₂O and the DNA concentration read in a nano-drop.

Shearing efficiency was checked by comparing the size of DNA fragments after sonication for 3 minutes and 6 minutes by agarose gel electrophoresis. A 1% agarose gel was made by dissolving 1g agarose in 100ml heated tris-acetate-EDTA (TAE) buffer and adding SybrSAFE ethidium bromide

substitute. This was poured into an electrophoresis tank tray with comb inserted and allowed to set for 30mins. 400ml TAE buffer was then added and the comb removed. The samples were prepared by adding 2µl Gel Pilot 5x loading dye (Qiagen) to 8µl sample and loaded into the gel. They were run at 100V alongside a DNA ladder (Direct Load 1kbp Ladder, Sigma) for 1h. Results are shown in Figure 3.9.

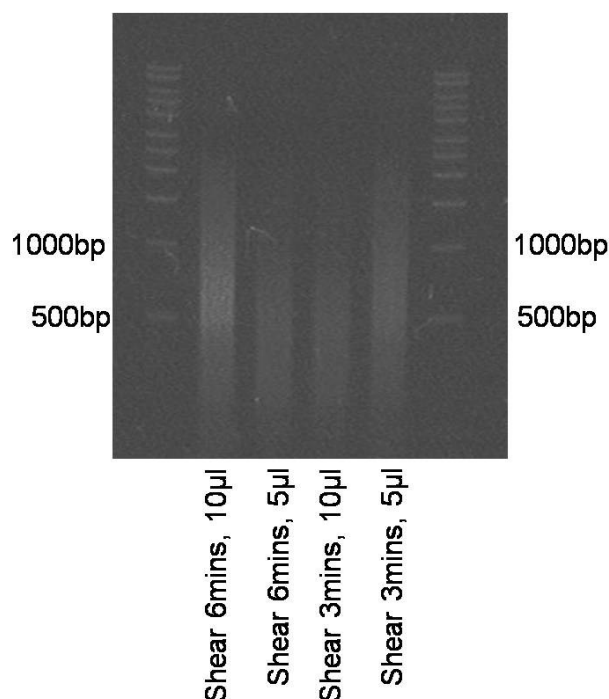


Figure 3.9 ChIP shearing optimisation DNA smear. Both 3 minutes and 6 minutes shearing by sonication yielded chromatin fragments around 500bp in size that would be suitable for ChIP. Bp: base pairs.

Immunoprecipitation and elution of chromatin

10µl chromatin was removed from each sample and stored for use as the input DNA control in the qRT-PCR analysis. ChIP reactions were set up with 150µl chromatin added to protein G magnetic beads, kit buffer 1, PIC, ddH₂O and 2µg anti-CREB antibody (Cell Signaling Technology) or normal rabbit IgG control (Cell Signaling Technology). Maximum chromatin was used in each reaction. The reactions were incubated on an end-to-end rotator at 4°C overnight, then washed 3 times using a magnetic stand in kit buffers to remove all unbound chromatin. The washed beads were resuspended in AM2 elution buffer and incubated on the rotator for 15 minutes at room temperature. The cross-links which joined pulled-down chromatin to antibody or matched IgG were reversed using the kit reverse cross-linking buffer, the magnetic beads were pelleted using the magnetic stand and the supernatant containing the chromatin of interest taken off. The ChIP and input DNA samples were then incubated with proteinase K for 1h before addition of proteinase K stop solution prior to qRT-PCR analysis.

qRT-PCR analysis

Analysis of the SOD2 promoter sequence (GenBank accession number AF059197) using MatInspector software[392] identified 9 putative CREB binding sites: 3 on the 5'-3' strand and 6 on the complementary strand (Figure 3.10). Of these, two were strong binding sites with one full CRE and one half site (see Figure 6.8). Primer-BLAST was used to design primers around these two sites with amplicons 100-200bp in length, as well as a negative control site in the SOD2 gene (GenBank accession number S77127) around 2000bp downstream of the transcription start site as a control for promoter binding. These were tested against human genomic DNA (prepared from HUVEC by Dr G Birdsey) to ensure a single PCR product when run in the thermocycler at 60°C. Primer sequences are shown in Table 3.7.

A qRT-PCR was then run using 5µl undiluted chromatin or input DNA diluted 1/10 from each condition, 1µl of primer working solution (forward and reverse primer diluted 1/10), 12.5µl SYBRgreen ready mix and ddH₂O to make each reaction volume 25µl. Each condition was run in triplicate. The thermocycler protocol was the same as that used for the standard qRT-PCR described in section 3.2.3. Results were analysed by normalising each sample to input DNA (after correction for the 1/10 dilution) and to the IgG control.



Figure 3.10 MatInspector readout of CREB-1 binding sites on the SOD2 promoter.

Primer Site	Sequences
1	Forward 5'-TGC AAA TCC TGC CTG CAG TCT C-3' Reverse 5'-GCG CCG TAC CCT TGC TTT GC-3'
2	Forward 5'-GTG CCA GAC CAC CTT GCC TGA-3' Reverse 5'-AAC GCA GAC AAG AGC AGG GGT-3'
Downstream	Forward 5'-GGC ACA CCG TTG TGG TGT CGT-3' Reverse 5'-AAC AAG CCT GTT TCA GTT TCC CAC T-3'

Table 3.7 Primer sequences used for ChIP RT-PCR for MnSOD promoter.

3.3 *In vivo* studies

3.3.1 Breeding of WBF1 cross and animal husbandry

In vivo experiments were carried out in accordance with Home Office guidelines (Project Licence PPL 70/6722) and with attention paid to the principles of reduction, refinement and replacement. Experiments were planned and carried out in a single block to minimise breeding of excess animals; they were performed once the majority of the *in vitro* work was completed in order to best target the *in vivo* experiments; and multiple tissue and serum samples were obtained at sacrifice from each animal. These measures kept the number of animals used and number of regulated procedures required to a minimum.

In order to generate male progeny of New Zealand white (NZW) female mice and BXSB male mice, breeding trios were set up containing 2 female NZW (Harlan) and 1 male BXSB (bred in-house with thanks to Miss J Rose). Litters were sexed and weaned at 21-26 days, with stock kept as separate sexes. Mice were housed in individually ventilated cages, supplied with standard food and water *ad libitum*, at 21°C with a 12 hour light/dark cycle. From 8 weeks onwards male animals were monitored twice weekly for signs of illness, and four times weekly from 12 weeks. Female animals were monitored twice weekly throughout. Animals showing signs of illness or distress were euthanased. Animals were sacrificed at 12, 16 and 24 weeks by CO₂ inhalation.

3.3.2 Treatment of cross with MTX

From 12 weeks until 16 weeks WBF1 male mice were injected weekly intraperitoneally with 1mg/kg MTX (TEVA) or 0.9% saline. The dose was chosen on the basis of literature reports of the MTX dose required to treat collagen-induced arthritis[393, 394] and of the dose needed to elicit changes in intracellular AICAR concentration[376] i.e. an anti-inflammatory rather than cytotoxic dosing schedule. Animals were numbered sequentially with alternate animals receiving MTX or 0.9% saline, to ensure littermates were used as controls and a coding system was used to maintain blinding of the investigators. They were weighed at 12 weeks for dose calculation. Total injection volume was 100µl.

3.3.3 Serum collection and storage

After sacrifice, blood was collected from experimental animals by cardiac puncture into the right atrium or into the inferior vena cava, aiming to extract 500µl. It was placed on ice in a 1.3ml microtube (Sarstedt) and allowed to stand at 4°C for 1-4h to allow coagulation, then centrifuged at 13000 rpm for 10 minutes. The serum was aspirated and re-spun for 5 minutes, then aliquoted and stored at -80°C.

3.3.4 Post mortem examination and harvesting and preparation of solid organs

The heart and descending aorta were harvested when the animals were sacrificed and a thorough inspection of all other organs was carried out. After confirmation of death, each animal was weighed and the abdominal and chest cavities were opened through a longitudinal incision from symphysis pubis to manubriosternal joint. Intrathoracic or peritoneal blood, ascites or pleural fluid were looked for, and the bowel, liver, spleen, kidneys and heart were examined. The heart was mobilised and removed by cutting through the ascending aorta. The descending thoracic aorta was then carefully mobilised and removed following an incision as close to the diaphragm as possible. The heart was fixed overnight in 10% buffered neutral formalin, then washed in PBS and placed in 70% ethanol. The following day they were paraffin embedded (with thanks to Ms L Lawrence). Hearts were bisected in a transverse plane at a mid-ventricular point, and both sections embedded side by side. 5µm sections were cut on a microtome and stained with hematoxylin/eosin (H&E) and periodic acid-Schiff (PAS) by Ms L Lawrence.

3.3.5 Preparation of aortae for immunoblotting and frozen sections

The descending aorta was harvested when the animal was sacrificed and placed in physiological saline solution on ice (see Appendix for recipe). It was dissected under microscope to strip off fat and connective tissue and then divided horizontally. At this point, aortic aneurysms were recorded if present. One half was immediately snap frozen in liquid nitrogen before storage at -80°C. The other half was embedded in OCT embedding compound (CellPath) on a cork board. A large drop of OCT was put on a small square cork board and the aorta section was laid lengthwise along one arm of small straight arm tweezers. It was then thrust into the OCT and immediately immersed in supercooled 2-methylbutane (Sigma) for 30 seconds. The tweezers were then removed leaving the aorta embedded upright, and the tissue stored at -80°C.

For immunoblotting, snap frozen aortic tissue was ground to a powder using a plastic pestle (Sigma) fitted into a 1.5ml eppendorf tube, cooled in liquid nitrogen. It was then dissolved in 100µl RIPA buffer supplemented with complete protease and phosphatase inhibitors, and incubated for 30 minutes on a rotator at 4°C, before centrifugation at 14000rpm for 15 minutes. The supernatant was collected and stored at -80°C for use in immunoblots.

Transverse sections of descending aorta embedded upright in OCT were cut on a Leica cryostat at 10µm thickness, and mounted onto Superfrost plus charged slides (Thermo Scientific). These were airdried at room temperature before fixing in acetone (for immunofluorescence) for 10 minutes and stored dry at -70°C.

3.3.6 Scoring of WBF1 arterial vasculopathy

The vasculopathy of the intramyocardial arteries of WBF1 mice was quantified in two ways, based on the scoring system described by Berden *et al*[395] and designed by me in conjunction with Dr J Boyle, consultant cardiovascular histopathologist. Paraffin-embedded PAS-stained myocardial sections were used. Firstly, peri-adventitial leukocytes surrounding each intramyocardial artery and arteriole were counted on a single section per animal. Secondly, PAS staining of arterial walls was scored as follows: 0: normal staining; 1: increased basement membrane staining; 2: increased basement membrane staining and nodular deposits. This was applied to every intramyocardial artery and arteriole in a single section per animal (approximately 10-15 vessels per section were examined in this way) and a mean score per animal generated.

3.3.7 Murine aortic ICAM-1 staining by immunofluorescence

Frozen transverse sections of murine aorta were dried at room temperature for 30 minutes, washed in PBS and blocked in 3% BSA/PBS at room temperature for 30 minutes. The rat anti-mouse ICAM-1 monoclonal antibody YN-1 (generated in-house), diluted to 10µg/ml in 3% BSA/PBS, was added to each slide (or rat IgG_{2a} isotype control (BD Pharmingen)) and incubated for 45 minutes. The slides were washed three times in PBS, then incubated with an Alexa Fluor 546-conjugated goat anti-rat IgG secondary antibody (Invitrogen Life Technologies), diluted to 8µg/ml in 3% BSA, for 45 minutes at room temperature in the dark. A directly conjugated Alexa Fluor 488 rat anti-mouse CD31 monoclonal antibody (MEC13.3; Biolegend, San Diego, USA) was used as an endothelial marker, and DRAQ-5 as a nuclear stain. Slides were incubated with these for 45 and 10 minutes respectively, before mounting with Fluoromount-G (Southern Biotech, Birmingham, Alabama), and visualisation by confocal microscopy. Quantification of staining was carried out using the LSM 510 META software. The endothelial or adventitial area was identified using the co-localised image incorporating anti-CD31, and the mean fluorescence intensity of the area recorded.

3.3.8 Calculation of myocardial infarct area

Cardiac sections stained with H&E were studied by light microscopy for evidence of infarcts. The sections were coded to ensure blinding of the investigator to the animal's treatment, and were examined independently by a second investigator to ensure agreement as to the number of affected animals. A single slide per animal was examined.

Acute infarcts were clearly visible on H&E stained slides. Older, fibrotic infarcts were imaged using picrosirius red to stain collagen. Cardiac sections were deparaffinised in Histoclear (National Diagnostics), then hydrated through a graded alcohol series to distilled water. Picrosirius red staining was carried out using a picrosirius red stain kit (Polysciences Inc), followed by dehydration in graded alcohol, brief clearing in Histoclear and mounting using Clarion Mounting Medium (Sigma).

Quantification of infarct area was carried out in two ways. Firstly, a single cardiac section from each animal was photographed with 8 fields of view per section, using the x10 objective, and the collagen area determined by thresholding using Image J software. In each photograph, a selection was made of infarct area and a histogram generated of intensity/ number of red pixels using the green image. This was compared to a selection representing background levels, to derive a cutoff above which any pixel was deemed to represent pathological collagen staining.

Secondly, photomicrographs were taken of each infarcted area to cover the whole infarct at x40 magnification, and the total area measured in Image J. Collagen morphometry with thresholding to determine the background cutoff was used to calculate the area of fibrotic infarcts as previously described, and acute infarcts were measured using a straightforward area measurement.

3.3.9 Murine serum cardiac troponin I ELISA

A high-sensitivity murine serum cardiac troponin I (cTnI) ELISA kit was purchased from Life Diagnostics UK Ltd. Murine serum samples (diluted 1:2 using the kit cTnI diluent) and serial dilutions of a murine cTnI standard supplied with the kit were pipetted in duplicate into microtitre wells coated in solid-phase anti-cTnI, along with a second HRP-conjugated anti-cTnI antibody in solution. The plate was incubated for 1hr on a rocking platform at room temperature, then washed using an automated plate washer (Thermo Scientific) to remove unbound HRP-conjugated antibodies and serum. A tetramethylbenzamine solution (an HRP substrate) was added and the plate was incubated for a further 20 minutes, resulting in the development of a blue colour proportional to the amount of bound cTnI. The colour development was stopped by the addition of a 1N HCl stop solution, resulting in a colour change to yellow. Absorbance at 450nm was then read on a plate reader, with concentration of cTnI proportional to absorbance. The standard curve was plotted using Microsoft Office Excel 2007, and the sample concentrations determined.

As the blood samples were obtained by puncture into the right atrium, an initial run of the ELISA was performed using serum samples obtained from two six month old female C57/Bl6 animals being used in another experiment. Paired blood samples were obtained from the inferior vena cava (IVC) and right atrium.

3.4 Statistical analyses

Data are expressed as the mean of at least 3 experiments, with error bars representing the standard error of the mean. All experimental results were analysed using Graphpad Prism 4 software.

Normalised data was analysed using Pearson's test for normality, followed by a single sample t-test, or Wilcoxon Rank Sum if the data was not normally distributed. Other parametric data was analysed by one-way or repeated-measures analysis of variance (ANOVA) or t-tests. Non-parametric data was

analysed using Mann-Whitney U test or Fisher's exact test. Results were considered significant if the p-value was less than 0.05.

4 HUVEC CHARACTERISATION AND RESPONSES TO VEGF AND HIGH FLOW LSS

4.1 Introduction

The aim of the work presented in this thesis was to establish how best to reduce atherosclerosis in the setting of chronic inflammatory disease, and to do so by investigating how to improve endothelial defence against inflammatory injury. The majority of the experiments were carried out *in vitro* using HUVEC isolated in the laboratory, and so initial experiments characterised these cells to ensure that those used were indeed endothelial. As high flow LSS and VEGF are powerful exogenous inducers of cytoprotective responses in adult vascular endothelium, these were investigated *in vitro* to look for evidence of synergy in the induction of cytoprotective genes.

4.1.1 Human umbilical vein endothelial cells

The majority of the experiments performed in this project were carried out on multiple isolates of HUVEC, used at an early passage (2-4). Using primary cells from multiple individuals has the advantage of representing population variability in genetic background, unlike a cell line, but there are a couple of drawbacks. First, it is possible to isolate smooth muscle cells and fibroblasts from the umbilical vein in addition to EC. Secondly, EC grown in culture over multiple passages can lose their endothelial-specific phenotype and de-differentiate. Therefore, in order to establish that the cells isolated were indeed endothelial in origin and maintained an endothelial phenotype for the duration of the experiments, staining with both constitutively expressed and inducible EC surface markers was carried out.

Vascular endothelium is a highly heterogeneous tissue, and EC have different properties depending on both the tissue they are resident in, and the type of vessel they line. This has implications for experimental practice, as HUVEC will not develop atherosclerosis. EC differentiation is stimulated and maintained by contact with neighbouring cells (both endothelial and pericytes); including via direct cell-cell adhesion through tight junctions, release of soluble mediators, and via the synthesis and organisation of the matrix proteins that form the endothelial basement membrane[396]. Many of these signals are rapidly lost when EC are grown in culture, which entails huge disruption in basement membrane, confluence, and loss of the tissue milieu of other cell types.

4.1.2 Endothelial cell characterisation

Confirming that the isolated cells are endothelial can be achieved in a number of ways. They can be stained for constitutive and inducible cell surface proteins that are specific to EC. They can be examined under microscopy for structures unique to EC such as Weibel-Palade bodies. Finally, they can be exposed to shear stress to mimic blood flow and their responses observed.

Constitutively expressed EC surface proteins include platelet-endothelial cell adhesion molecule (PECAM-1; CD31), ICAM-2 and endoglin. A list, along with the other cell types that express them, is shown in Table 4.1. CD31, a member of the immunoglobulin superfamily, is expressed on EC mostly at intercellular junctions, where it is the principal component[397]. In addition it forms part of an EC mechanotransduction complex and is essential for altering intracellular signalling based on the shear stress applied to EC surface[147]. ICAM-2, a transmembrane glycoprotein and another member of the immunoglobulin superfamily, binds LFA-1 on leucocytes. It is constitutively expressed at a much higher level than ICAM-1 on resting EC, and is found principally at intercellular junctions, where it is important for mediating leucocyte trafficking[398]. Endoglin, also a transmembrane glycoprotein, forms part of the TGF β receptor complex. In resting EC it is constitutively expressed at low levels, but is much more highly expressed on proliferating cells and thus on those grown in culture[399].

Adult vascular endothelium can be activated by a variety of cytokines and growth factors resulting in extensive changes in gene expression, which can be reversible[400]. Responses to growth factors in particular can be very different depending on whether the cells are confluent and securely attached to their neighbours via tight junctions. Cell-cell contact inhibits both spontaneous and growth factor-induced cell growth and division, and other key endothelial properties are also changed: e.g. arachidonic acid metabolism, and release of lytic enzymes and growth factors[396]. Inducible EC surface proteins include DAF, which is induced by VEGF via ligation of VEGFR2 and a pathway that includes PKC ϵ and phospholipase C γ [176], and the cell adhesion molecules ICAM-1, VCAM-1 and E-selectin which are induced by pro-inflammatory cytokines such as TNF α and IL-1 via activation of NF κ B[401]. Table 4.2 illustrates the array of inducible cell surface proteins expressed by vascular endothelium.

Antigen	Cell Type
PECAM-1 (CD31)	EC, platelets, lymphocytes, monocytes, neutrophils
Angiotensin-converting enzyme	EC, epithelial cells, monocyte-macrophages, T lymphocytes
Type I scavenger receptor (acetylated-LDL uptake)	EC, macrophages, SMCs, pericytes, fibroblasts
<i>Ulex europaeus</i> I agglutinin binding / O(H) blood-type antigen	EC, erythrocytes
<i>Bandeirea simplicifolia</i> lectin binding	EC
<i>Griffonia simplicifolia</i> agglutinin binding	EC
V-E cadherin (CD144)	EC, trophoblasts, PLN sinus macrophages
CD34	EC, hemopoietic precursors
ICAM-2 (CD102)	EC, lymphocytes, monocytes, platelets
CD51/61 (vitronectin receptor)	EC, platelets, osteoclasts, mast cells, lymphocytes
Endoglin (CD105)	EC, monocyte-macrophages, B lymphocytes, syncytiotrophoblasts
CD36	Microvascular EC, monocyte-macrophages, erythroid cells, platelets
VAP-2	EC, lymphocytes, tonsillar epithelium
S-ENDO 1/MUC18	EC, SMCs, dendritic cells
Thrombomodulin	ECs, SMCs

Table 4.1 Constitutively expressed EC surface markers (adapted from Garlanda and Dejana[396]).

Antigen	Cell Type	Stimulus
CD54/ ICAM-1	ECs, leukocytes, epithelium, fibroblasts	Upregulated in ECs by inflammatory cytokines
CD106/ VCAM-1	ECs, macrophages, synoviocytes, dendritic cells, mesothelium	Upregulated in ECs by inflammatory cytokines
CD62E/ E-selectin	ECs, (postcapillary venules restricted in vivo)	Induced by inflammation, CK and neoangiogenesis
CD62P/ P-selectin	ECs, platelets	Membrane exposed after CK activation
VEGFR-2	ECs	Angiogenesis, KS, vascular tumors, laminar flow
VEGFR-1	ECs	Angiogenesis, KS
Tie-1	ECs	Angiogenesis
Tie-2/Tek	ECs	Angiogenesis
DAF (CD55)	EC	Statins, VEGF
VAP-1	EC	

Table 4.2 Inducible EC surface markers (adapted from Garlanda and Dejana [396]).

4.1.3 High flow LSS and VEGF

Blood vessel walls exposed to long term high flow laminar shear stress (LSS) are resistant to the development of atheroma, which occurs mainly at branch points and other sites of low or turbulent flow[143]. LSS conditions the vascular endothelium to adopt an anti-inflammatory, anti-apoptotic, quiescent phenotype, through effects on gene expression. Similarly, the endothelial growth factor VEGF also alters gene expression to promote survival and enhance endothelial defence against injury[174, 177]. The ways in which LSS and VEGF encourage endothelial cytoprotection were discussed in Section 1.4.

Synergy, the co-operative action of two or more stimuli to result in a response that is different or greater than the sum of the actions together, is an important biological phenomenon. There are many examples of synergy between stimuli acting on EC to produce an enhanced response: treating EC with atorvastatin and rapamycin together results in a synergistic induction of DAF[206]; and treating EC exposed to LSS with atorvastatin results in a synergistic induction of HO-1[248]. This last paper indicates that LSS alters the way in which EC respond to other exogenous mediators, which may explain its effects on atheroma development.

This chapter addresses the hypothesis that exposing EC to LSS and VEGF together results in synergistic induction of cytoprotective genes. Understanding how such a mechanism might work would be important for addressing how best to tackle atheroprotection pharmacologically.

There is some evidence to suggest that LSS and VEGF may interact to enhance endothelial cytoprotection. Firstly, LSS induces expression of VEGFR2, and indeed is capable of signalling through it[147, 402]. VEGFR2 is the principal endothelial VEGF receptor. In addition to mediating VEGF signalling, however, it also forms part of the endothelial mechanosensing and mechanotransduction system for transmitting the effects of shear stress intracellularly. It forms a complex with VE-cadherin and PECAM-1, and is activated very rapidly by the onset of laminar flow[147].

Secondly, VEGF and LSS both activate eNOS and enhance NO production, an action that requires phosphorylation and activation of VEGFR2[148, 403]. They also both enhance expression of prostacyclin[171, 404].

Thirdly, both LSS and VEGF can induce expression of HO-1, a highly inducible enzyme that has well-established cytoprotective properties, discussed in Section 1.4. It is readily induced by LSS[140, 248], and by VEGF[405-407]. It should be noted however that those papers showing induction of HO-1 by VEGF do so in the context of angiogenesis, where cell-cell contact is very different to that in confluent monolayers. This change in EC environment may have significant effects on the characteristics of the response to VEGF.

Finally, there is the question of whether LSS induces VEGF expression itself by EC. The evidence for this is conflicting. Passerini *et al* reported a 3 fold increase in VEGF mRNA expression in HUVEC exposed to LSS at 15 dynes/cm² for 24h[408]. However, other studies have reported a down-regulation in VEGF expression, albeit after shorter periods of exposure to flow[409]. Conklin *et al* reported enhanced expression of VEGF in porcine carotid arteries exposed to low shear stress for 24h, interpreting this as a reason for increased endothelial permeability to lipid at regions in the arterial tree of low or turbulent flow[410].

4.1.4 Chapter Aims

The aims of the experiments presented in this chapter were, firstly, to establish that the cells isolated and used for the project were indeed endothelial in origin; and, secondly, to investigate synergy in the actions of VEGF and LSS on HUVEC, using the induction of HO-1 as a model. The effects of LSS and VEGF on the induction of a wider selection of genes linked with cytoprotection was then explored. These were either genes known to be induced by VEGF (such as DAF, BCL-2 or A1), or those thought to be regulated by both (VEGFR2, eNOS). Lastly, a phosphokinase array was used to explore the signalling pathway changes that occurred following VEGF stimulation under static or LSS conditions.

4.2 Results

4.2.1 Staining of constitutively expressed EC surface markers

Passage 3 HUVEC were stained with antibodies to the constitutively expressed proteins PECAM-1 (CD31), ICAM-2 and endoglin and the percentage of positively stained cells was analysed by FACS. 97.7% of cells stained with anti-CD31 (Figure 4.1A), 96.7% with anti-ICAM2 (Figure 4.1B) and 72.6% with anti-endoglin (Figure 4.1C), indicating that a high percentage of the cells in culture are indeed endothelial in origin.

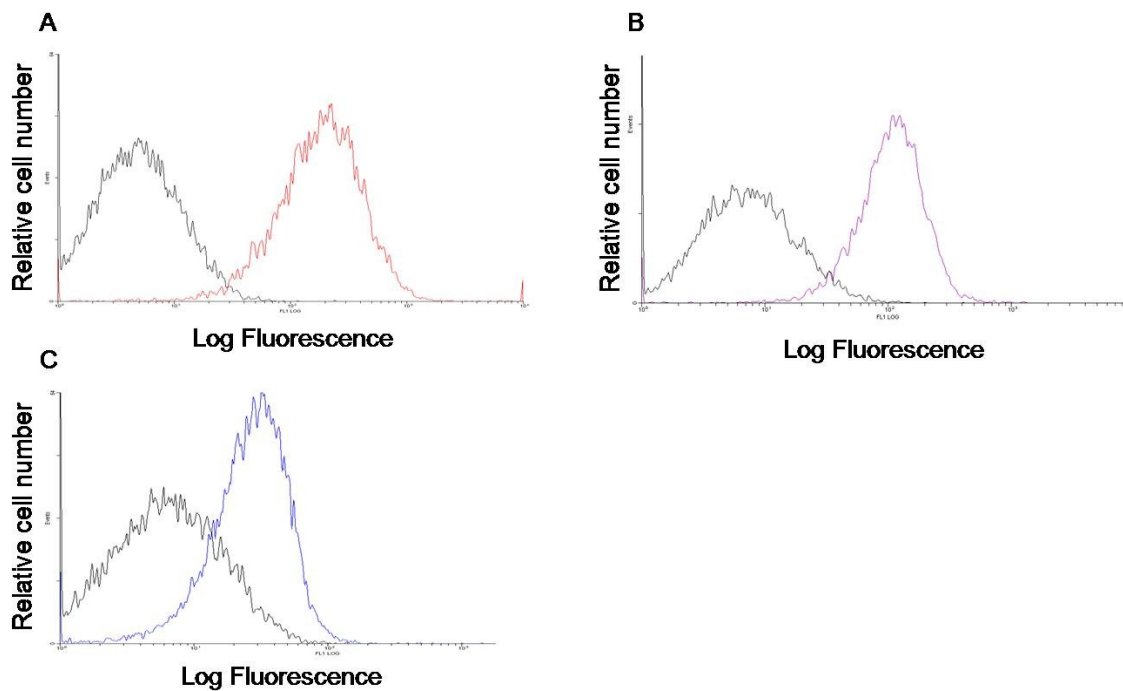


Figure 4.1 HUVEC stain with antibodies specific to EC surface proteins. HUVEC were stained with antibodies to CD-31, endoglin and ICAM-2 and positive cells were counted by flow cytometry. A: black: unstained cells; red: anti CD-31 antibody. B: black: unstained cells; purple: anti-endoglin antibody. C: black: unstained cells; blue: anti-ICAM-2 antibody.

4.2.2 Inducible EC surface markers: DAF induction by VEGF

HUVEC were treated with VEGF 25ng/ml for 48h. Induction of DAF was measured by FACS, and a two-fold induction was observed (Figure 4.2), consistent with the published literature[176].

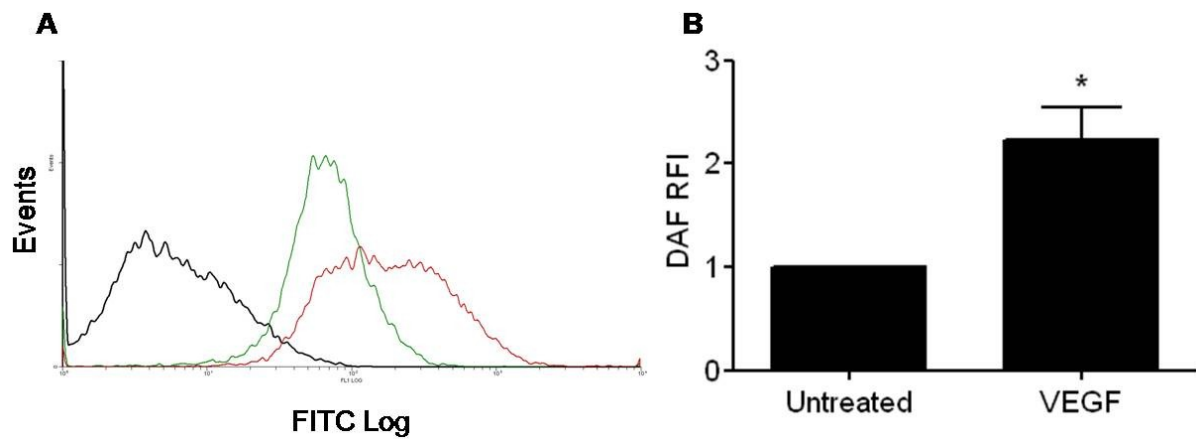


Figure 4.2 Induction of DAF expression in HUVEC by VEGF. HUVEC were treated with VEGF 25ng/ml x 48h, then stained with anti-DAF and analysed by flow cytometry. A: representative histogram. Black: unstained HUVEC; green: untreated HUVEC stained with anti-DAF antibody (1H4); red: HUVEC treated with VEGF 25ng/ml stained with anti-DAF antibody. B: histogram showing RFI of 3 experiments. Results are expressed as mean $\pm$ SE and represent at least 3 experiments. * = $p < 0.05$.

4.2.3 Inducible EC surface markers: ICAM-1 and VCAM-1 induction by TNF α

HUVEC were treated with TNF α 1 and 10ng/ml for 16h, then stained with anti-ICAM-1 and anti-VCAM-1 and their expression levels were measured by FACS. A 12-fold induction of ICAM-1 and 15-fold induction of VCAM-1 was observed (Figures 4.3 and 4.4 respectively), consistent with the published literature.

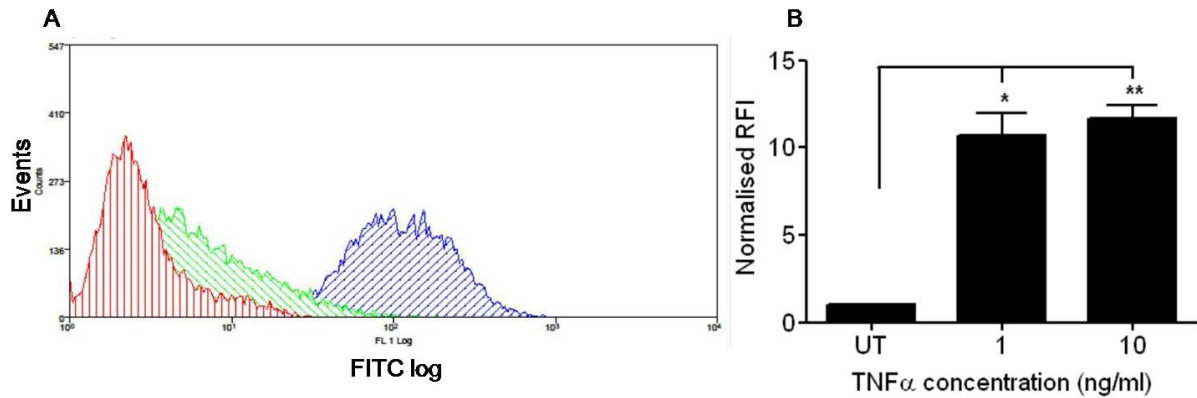


Figure 4.3 Induction of ICAM-1 by TNF α . HUVEC were treated with TNF α 1ng/ml or 10ng/ml for 16h, then stained with anti-ICAM1 antibody and analysed by flow cytometry. A: representative histogram; red: unstained cells; green: untreated cells stained with anti-ICAM-1 antibody; blue: cells treated with TNF α 10ng/ml stained with anti-ICAM-1 antibody (6.5B5). B: histogram showing normalised RFI of 3 experiments. Data are presented as mean $\pm$ standard error. * = $p < 0.05$; ** = $p < 0.01$

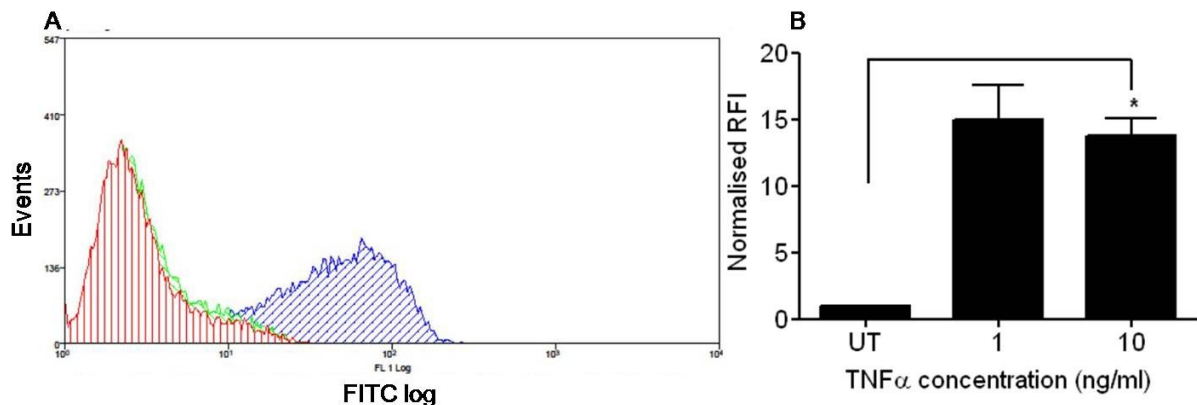


Figure 4.4 Induction of VCAM-1 by TNF α . HUVEC were treated with TNF α 1ng/ml or 10ng/ml for 16h, then stained with anti-VCAM1 antibody and analysed by flow cytometry. A: representative histogram; red: unstained cells; green: untreated cells stained with anti-VCAM-1 antibody (1.4C3); blue: cells treated with TNF α 10ng/ml stained with anti-VCAM-1 antibody. B: histogram showing normalised RFI of 3 experiments. Data are presented as mean $\pm$ standard error. * = $p < 0.05$.

4.2.4 Effect of VEGF on HO-1 expression in static-cultured HUVEC

Although induction of HO-1 by VEGF has been previously shown[405-407], I was unable consistently to demonstrate this (Figure 4.5). A trend towards induction of HO-1 mRNA at 8h was observed, and protein at 24h; however this failed to reach statistical significance. Changing cell confluence (50% vs 75%); medium conditions (varying concentrations of FCS) and cell type (HMEC were also tried) did not result in a consistent improvement in the induction seen. HO-1 protein expression was also analysed according to whether the untreated cells expressed high or low quantities of HO-1, but there was no relationship between this and response to VEGF. Cells with either high or low baseline HO-1 levels were capable of responding to VEGF in some cases, and not in others.

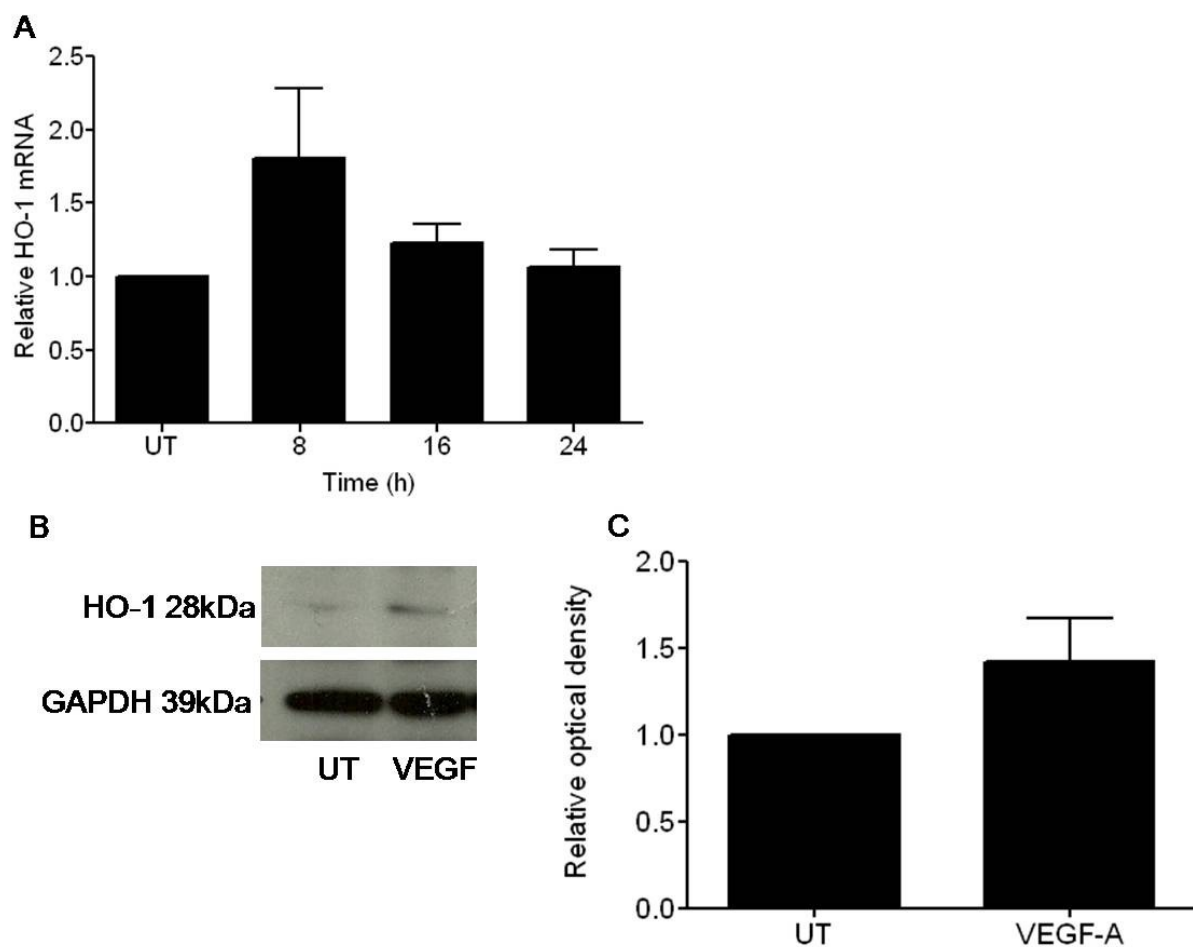


Figure 4.5 HO-1 mRNA and protein expression in HUVEC stimulated with VEGF. HUVEC were treated with VEGF 25ng/ml over a 24h time course then lysed and analysed by qRTPCR or by western blot. A: Time course of induction of HO-1 mRNA by VEGF 25ng/ml. B and C: HO-1 protein expression in HUVEC after stimulation with VEGF 25ng/ml for 24h. Data shown are the results of >9 experiments and are expressed as mean +/- standard error.

4.2.5 LSS induces HO-1 expression in HUVEC

The Cytodyne Streamer parallel plate apparatus was used to expose HUVEC grown on a fibronectin-coated slide to LSS 12-20 dynes/ cm² for 24h. Induction of HO-1 mRNA (Figure 4.6A) and protein (densitometry in Figure 4.6B; representative immunoblot shown in Figure 4.9) was confirmed, consistent with the published literature.

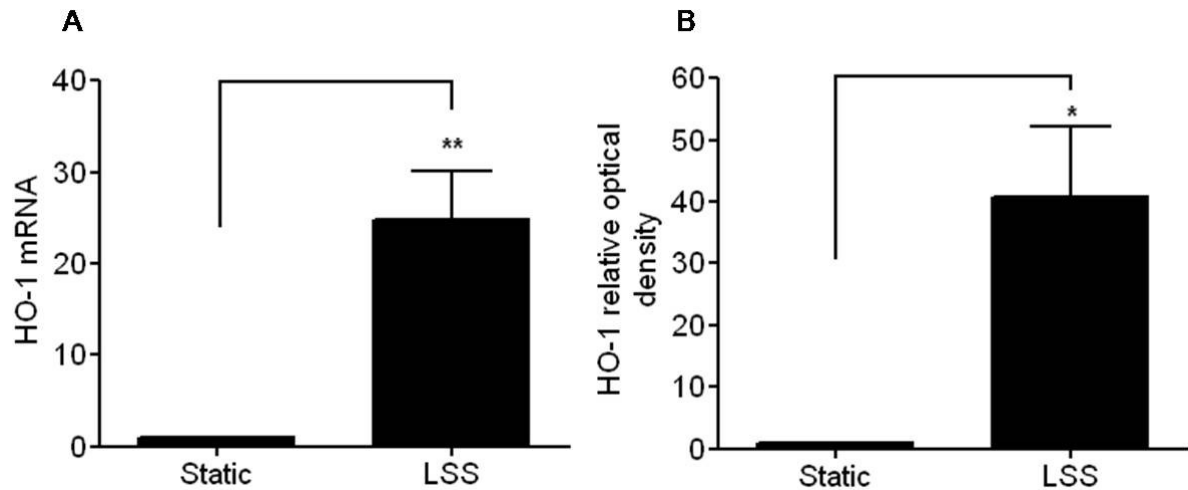


Figure 4.6 Induction of HO-1 mRNA and protein by LSS. HUVEC were exposed to LSS 20 dynes/cm² for 24h in the cytodine streamer parallel plate system, or static conditions in a Petri dish. A: induction of HO-1 mRNA. B: normalised densitometry results of induction of HO-1 protein. A representative blot is shown in Figure 4.9. Data shown are the results of 4 experiments and expressed as mean +/- standard error. * = p<0.05; ** = p<0.01.

4.2.6 Effect of VEGF and LSS on induction of HO-1

I hypothesised that exposure to LSS may sensitise vascular endothelium to VEGF, leading to enhanced induction of cytoprotective genes. In order to explore this, two cytodine flow circuits were set up simultaneously, and after allowing 16h equilibration to flow, VEGF 25ng/ml was added to one circuit, as well as to HUVEC kept in static conditions. Two time points were investigated to try to capture all effects of VEGF on gene transcription in HUVEC equilibrated to LSS: an early one (4h for mRNA and 8h for protein) and a late one (24h). These were chosen on the basis of experiments exploring VEGF-induced anti-apoptotic gene expression in confluent HUVEC and those previously investigating HO-1 induction by VEGF[175, 405]. HO-1 mRNA levels were measured after exposure to VEGF for 4h, and a significant increase in HO-1 mRNA was observed when VEGF was added to cells exposed to LSS, indicating a synergistic induction (Figure 4.7). However, this was not consistently observed at 24h where only 2/5 replicates followed this pattern (Figure 4.8). Furthermore, a synergistic induction of HO-1 protein by LSS and VEGF was not demonstrated (Figures 4.9 and 4.10).

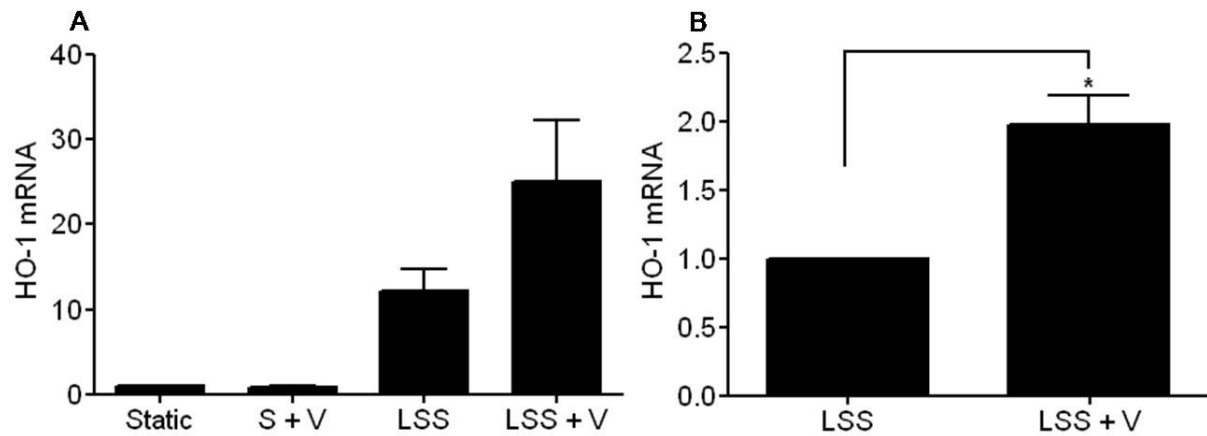


Figure 4.7 Induction of HO-1 mRNA by LSS and VEGF for 4h. HUVEC were exposed to static conditions or LSS 20 dynes/cm² for 16h, before the addition of VEGF 25ng/ml for 4h. A: comparison of results under static conditions, VEGF added under static conditions (S+V); LSS, and VEGF added to LSS (LSS+V). B: fold change in HO-1 mRNA induction when VEGF is added under LSS conditions. Data are expressed as mean +/- standard error and represent the results of 3 experiments. * = p<0.05.

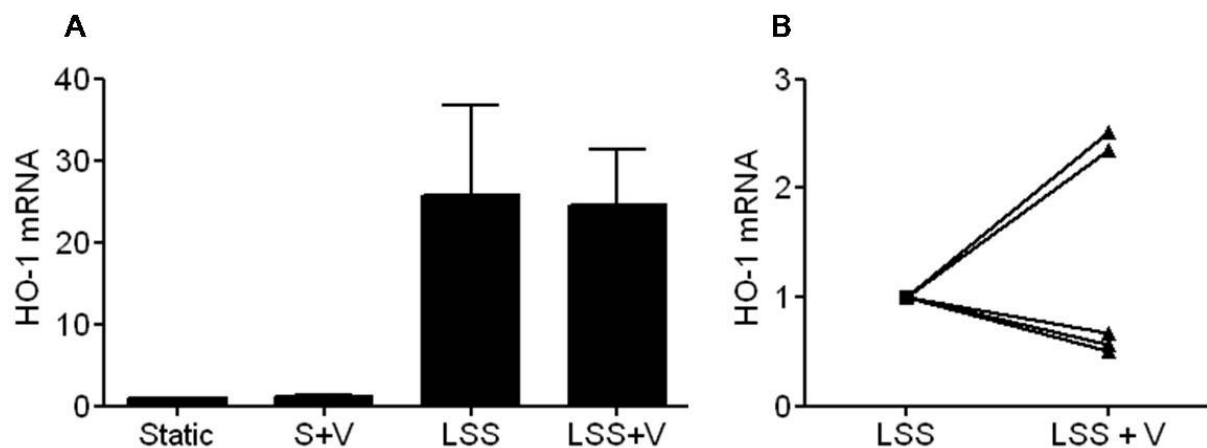


Figure 4.8 Induction of HO-1 mRNA by LSS and VEGF for 24h. HUVEC were exposed to static conditions or LSS 20 dynes/cm² for 16h, before the addition of VEGF 25ng/ml for 24h. A: comparison of results under static conditions, VEGF added under static conditions (S+V), LSS, and VEGF added to HUVEC exposed to LSS (LSS+V). B: fold change in HO-1 mRNA induction when VEGF is added under LSS conditions, demonstrating the variability in response between experiments. Data are expressed as mean +/- standard error and represent the results of 5 experiments.

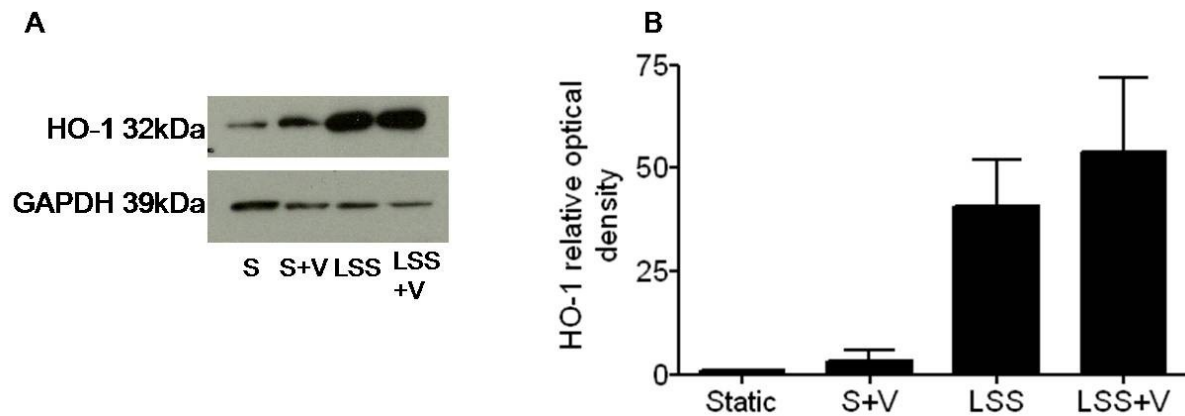


Figure 4.9 Induction of HO-1 protein by LSS and VEGF for 8h. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 8h. A: representative western blot. S: static conditions, S+V: VEGF 25ng/ml for 8h under static conditions, LSS: laminar shear stress, LSS+V: LSS and VEGF 25ng/ml for 8h (LSS+V). B: normalised densitometry quantifying change in HO-1 protein. Data are expressed as mean $\pm$ standard error and represent the results of 4 experiments.

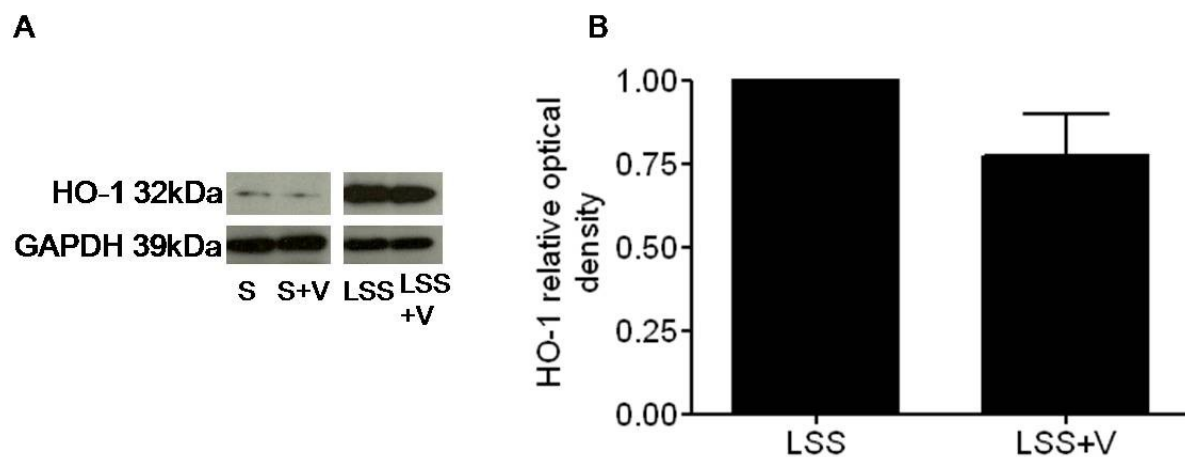


Figure 4.10 Induction of HO-1 protein by LSS and VEGF for 24h. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 8h. A: representative western blot. S: static conditions, S+V: VEGF 25ng/ml for 8h under static conditions, LSS: laminar shear stress, LSS+V: LSS and VEGF 25ng/ml for 8h (LSS+V). B: normalised densitometry quantifying change in HO-1 protein for cells exposed to LSS only. Data are normalised to LSS, are expressed as mean $\pm$ standard error and represent the results of 4 experiments.

4.2.7 Effect of VEGF and LSS on induction of other cytoprotective genes

VEGF and LSS induce many other anti-oxidant, anti-inflammatory and anti-apoptotic genes in addition to HO-1, but the effect of exposing HUVEC to both together *in vitro* has not been explored. I investigated the induction of eNOS, VEGFR2, DAF, A1, Bcl-2 and MnSOD mRNA in cells exposed to laminar shear stress or static conditions, then stimulated with VEGF for 4 or 24h.

Although serine 1177 phosphorylation of eNOS by VEGF is well documented[411], induction of eNOS mRNA expression by VEGF under static conditions did not occur at 4 or 24h (Figure 4.11). LSS did induce eNOS mRNA, consistent with the published literature[153]. The addition of VEGF to LSS conditions resulted in a down-regulation of mRNA at 4h, which had normalised at 24h.

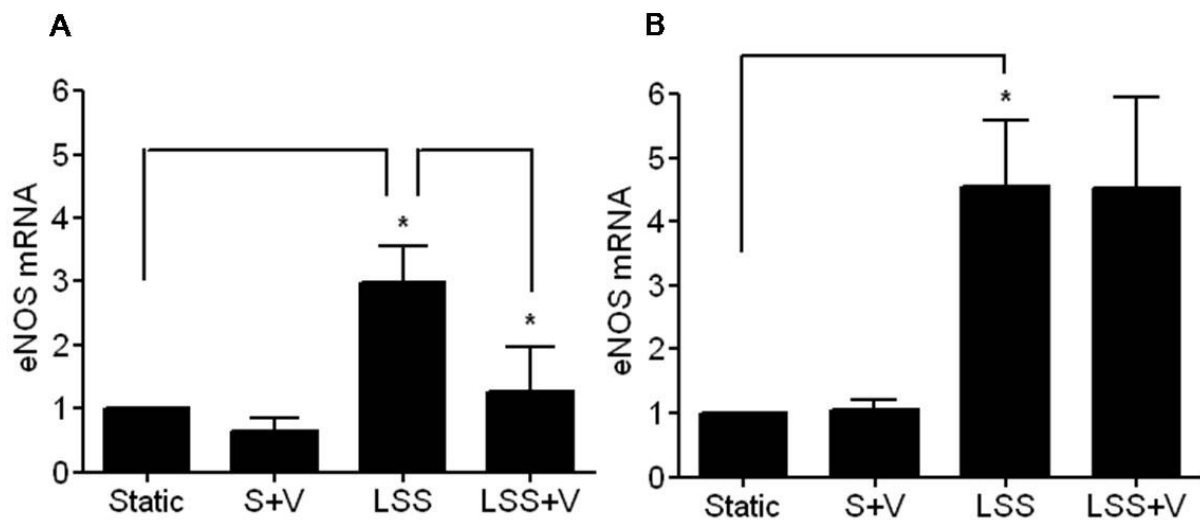


Figure 4.11 Induction of eNOS mRNA by LSS and VEGF. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 4h (A) or 24h (B). S+V: VEGF added in static conditions; LSS: laminar shear stress; LSS+V: VEGF added to LSS conditions. Data are expressed as mean $\pm$ standard error and represent 4 experiments. * = $p < 0.05$.

Again, consistent with the published literature, I observed a trend towards induction of VEGFR2 mRNA with LSS, although this did not reach statistical significance, and a small induction with VEGF stimulation for 24h under static conditions. No statistically significant change in VEGFR2 mRNA levels was observed with VEGF stimulation under LSS conditions (Figure 4.12).

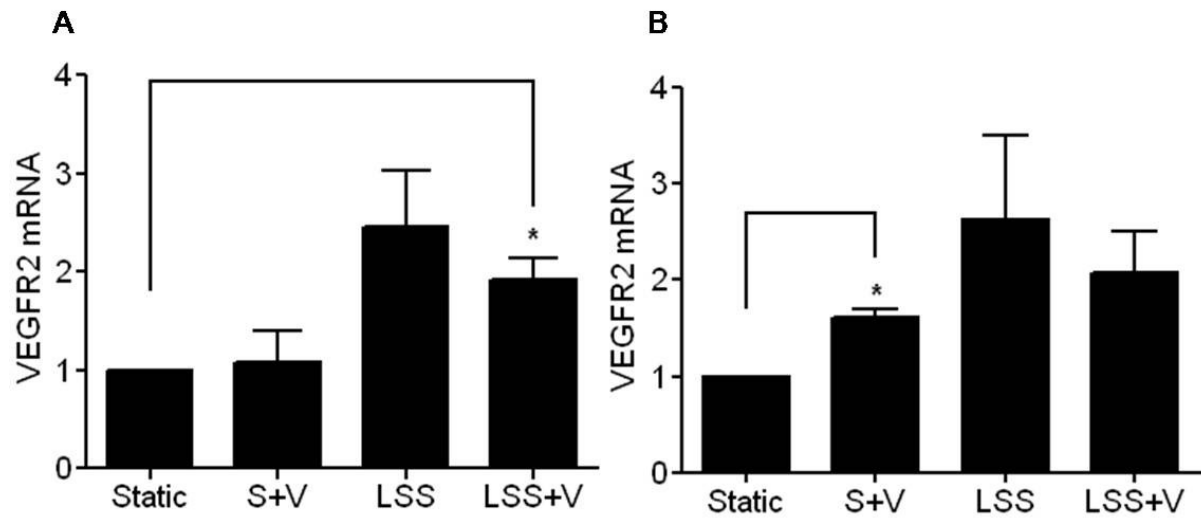


Figure 4.12 Induction of VEGFR2 mRNA by LSS and VEGF. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 4h (A) or 24h (B). S+V: VEGF added in static conditions; LSS: laminar shear stress; LSS+V: VEGF added to LSS conditions. Data are expressed as mean $\pm$ standard error and represent 4 experiments. * = $p < 0.05$.

DAF is a complement inhibitory protein which is induced in EC by VEGF. In my system, the expected induction of DAF mRNA with VEGF treatment under both static and flow conditions was observed, but the presence of LSS did not alter this (Figure 4.13).

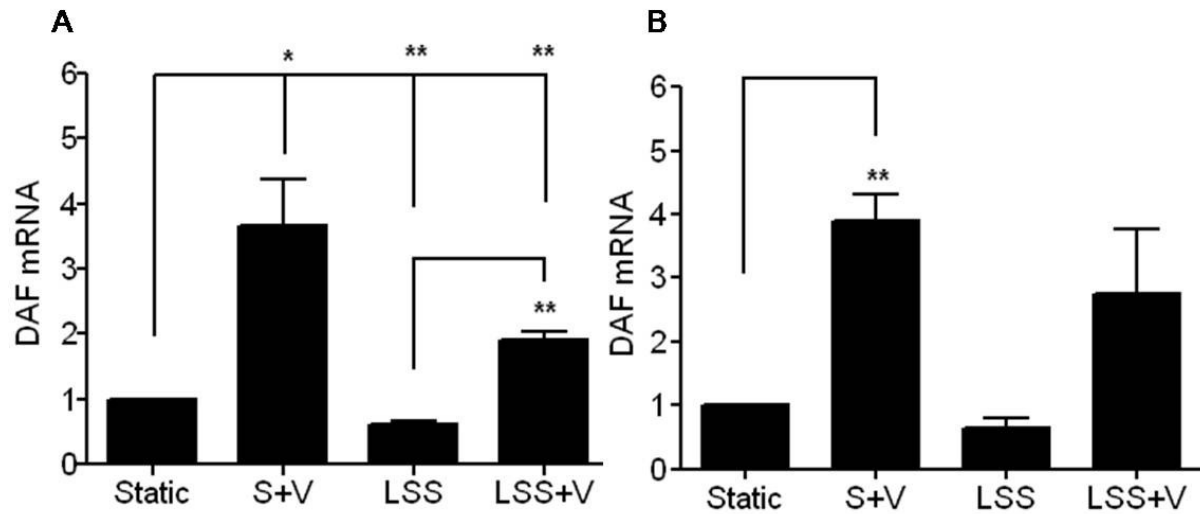


Figure 4.13 Induction of DAF mRNA by LSS and VEGF. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 4h (A) or 24h (B). S+V: VEGF added in static conditions; LSS: laminar shear stress; LSS+V: VEGF added to LSS conditions. Data are expressed as mean $\pm$ standard error and represent 4 experiments. * = $p < 0.05$; ** = $P < 0.01$.

A1 and Bcl-2 are two related anti-apoptotic proteins that have previously been shown to be induced in EC by VEGF[174]. No statistically significant increase in either was seen in static-cultured cells treated with VEGF, but this may have been to do with timing, as the optimal time point for induction of both is thought to be around 8h. These samples were studied at 4h and 24h. A1 mRNA was surprisingly significantly down-regulated by LSS for 24h, but this was a transient phenomenon and had recovered by 40h (Figure 4.14). However, induction of A1 mRNA by VEGF was likely to be preserved under LSS conditions at the short time point, although the large error bars prevented this reaching significance. No consistent effect of VEGF or LSS on Bcl-2 mRNA expression was observed (Figure 4.15).

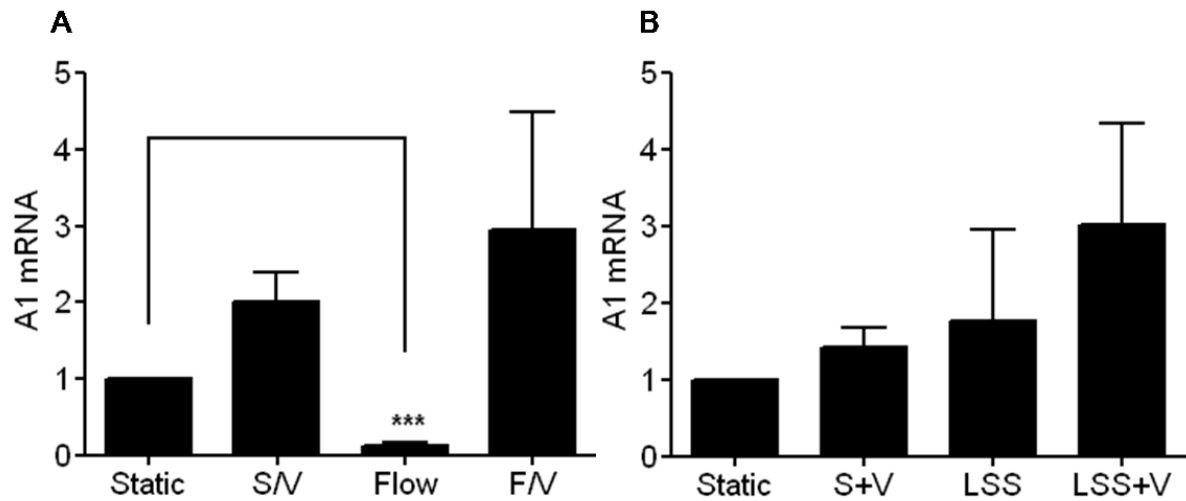


Figure 4.14 Induction of A1 mRNA by LSS and VEGF. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 4h (A) or 24h (B). S+V: VEGF added in static conditions; LSS: laminar shear stress; LSS+V: VEGF added to LSS conditions. Data are expressed as mean $\pm$ standard error and represent 4 experiments. *** = $P < 0.001$.

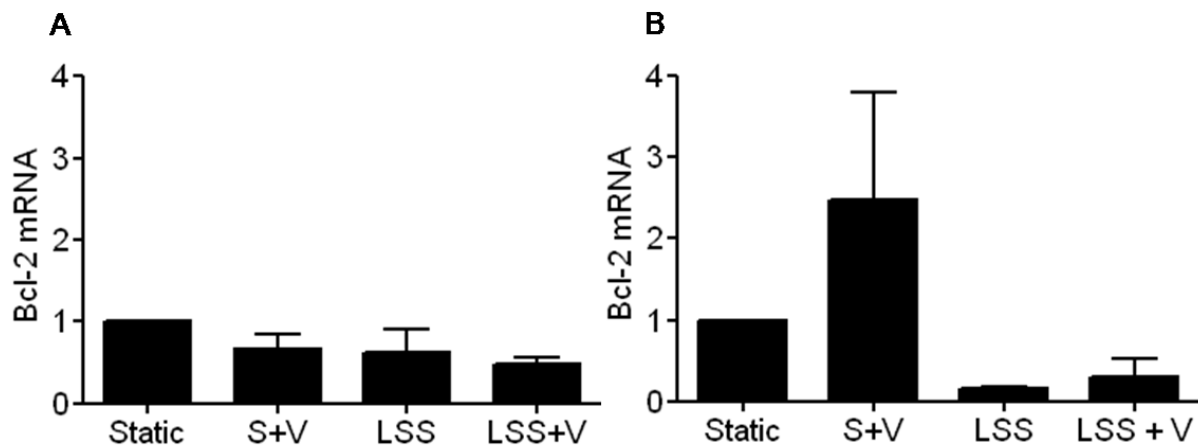


Figure 4.15 Induction of Bcl-2 mRNA by LSS and VEGF. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 4h (A) or 24h (B). S+V: VEGF added in static conditions; LSS: laminar shear stress; LSS+V: VEGF added to LSS conditions. Data are expressed as mean $\pm$ standard error and represent 2 experiments.

Finally, the effect of VEGF and LSS on MnSOD mRNA was studied. LSS did not induce MnSOD, but VEGF may do so under static conditions, although loss of samples meant that this did not reach significance. However, there was no potentiating effect on MnSOD mRNA expression of LSS and VEGF together.

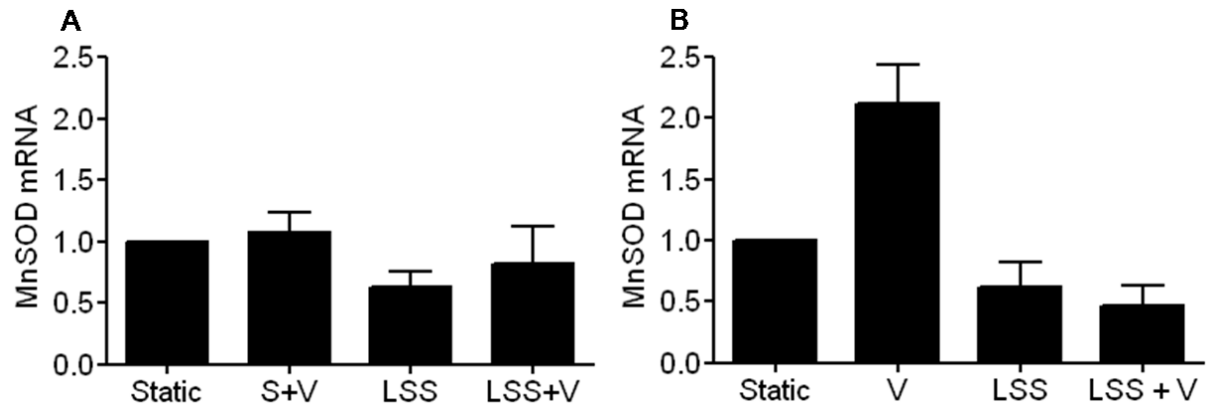


Figure 4.16: Induction of MnSOD mRNA by LSS and VEGF. HUVEC were exposed to static or LSS conditions for 16h, before the addition of VEGF 25ng/ml for 4h (A) or 24h (B). S+V: VEGF added in static conditions; LSS: laminar shear stress; LSS+V: VEGF added to LSS conditions. Data are expressed as mean $\pm$ standard error and represent 4 experiments (A) or 2 experiments (B).

4.2.8 Effect of VEGF under LSS conditions on protein phosphorylation

Given that no clear pattern of synergistic induction with VEGF and LSS was observed with any of the cytoprotective genes analysed earlier, a different approach was adopted to look for evidence in the Cytodyne system of synergy between VEGF and LSS. A phosphokinase array was used to screen for phosphorylation changes in HUVEC treated with VEGF under both static and LSS conditions.

Preliminary experiments established that VEGF induced strong ERK1/2 phosphorylation after 15 minutes. HUVEC were therefore exposed to static conditions or LSS for 24h, before the addition of VEGF 25ng/ml for 15 minutes, then immediate lysis on ice. Quantification of the results is shown in Figures 4.17, where results are expressed relative to the untreated static condition. In Figure 4.18, the effect of VEGF alone is shown by expressing the effects of VEGF on static-cultured cells relative to the untreated static control, and VEGF under LSS conditions expressed relative to LSS alone.

In the majority of cases, the addition of VEGF under LSS conditions resulted in reduced phosphorylation compared to VEGF addition to static cells (e.g. eNOS). Those phosphokinases exhibiting increased phosphorylation with VEGF and LSS together are summarised in Tables 4.1 and 4.2. Interestingly, several of these are thought to have roles in preventing cell proliferation (AMPK; p27), while VEGF is usually thought of as an endothelial mitogen[324, 412]. Although these changes were not subsequently validated and therefore no conclusions can be drawn, it does suggest that the influence of VEGF on confluent EC exposed to LSS is likely to be very different to that exerted when VEGF is used to stimulate static-cultured cells.

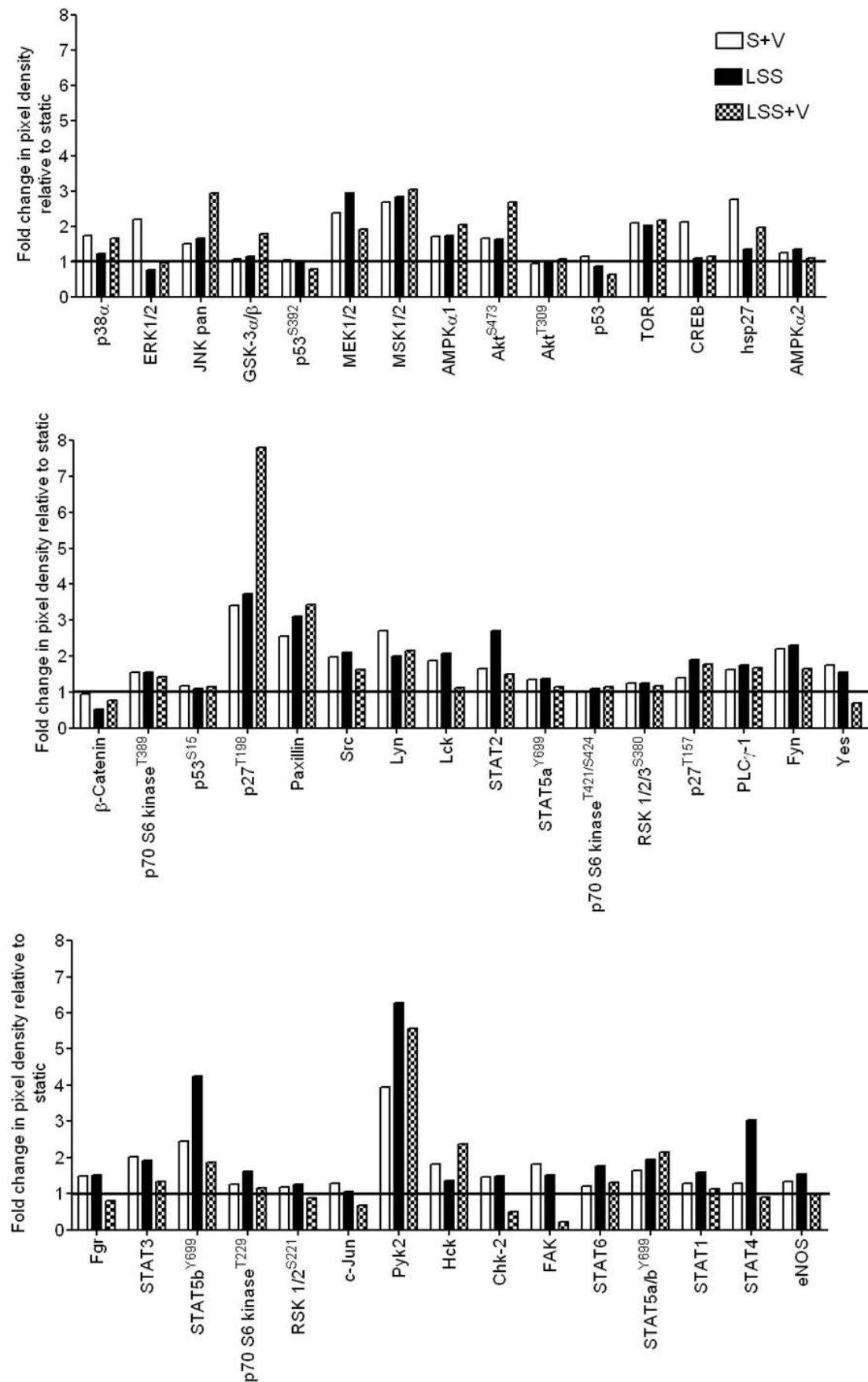


Figure 4.17 Phosphokinase array densitometry results presented with static-cultured cells as baseline.

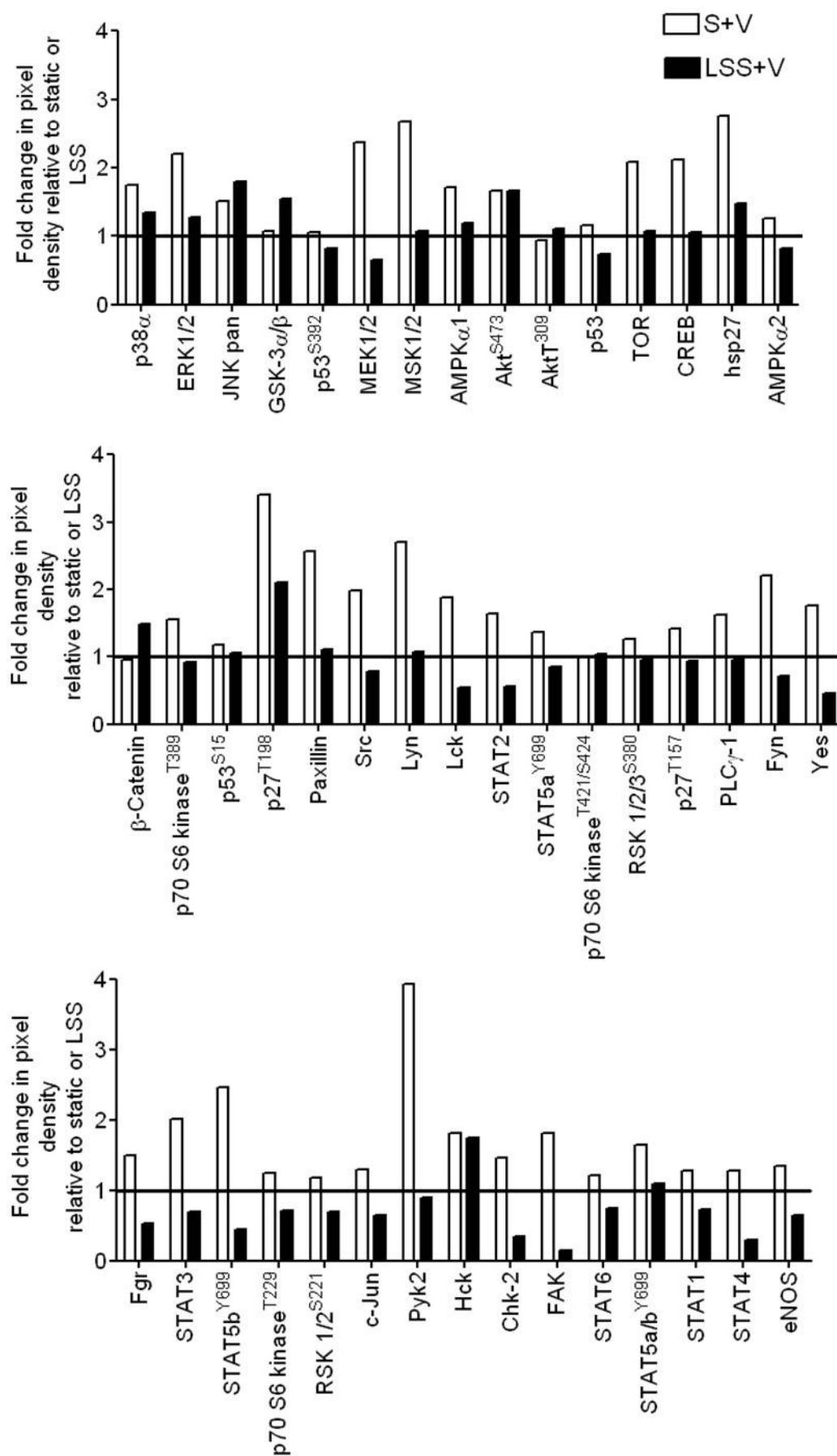


Figure 4.18 Phosphokinase array densitometry results presented relative to static or LSS-cultured HUVEC.

Phosphokinases exhibiting greater phosphorylation with VEGF under LSS than under static conditions	Functions in EC
JNK pan	Cell proliferation and migration; response to inflammatory mediators
GSK-3 α/β	Phosphorylation usually leads to deactivation of target proteins. Cell proliferation; migration. EC permeability in response to VEGF
Akt ^{Ser473}	Cell proliferation and survival. Mediates effects on metabolism of insulin signalling. May have role in angiogenesis
β -catenin	EC permeability in response to VEGF
Hck	SrcB subfamily tyrosine kinase. Little known about actions in endothelium

Table 4.3 Phosphokinases exhibiting increased synergy with VEGF and LSS together and their functions in EC.

Other phosphokinases activated by VEGF under both static and LSS conditions	Functions in EC
p38 α	Response to cytokines and stress. Increased EC permeability in response to VEGF
ERK1/2	Cell proliferation
AMPK α 1	Energy homeostasis; reduced cell proliferation; increases eNOS phosphorylation
Hsp27	Refolding proteins in times of stress. Downstream substrate of p38. Cell proliferation.
p27 ^{T198}	Cell cycle regulator. Blocks cell cycle progression. Role in cell motility

Table 4.4 Phosphokinases activated by VEGF in both static and LSS conditions and their functions.

4.3 Discussion

The results presented in the first part of this chapter show that the early passage HUVEC isolated for use in this project express CD31, ICAM-2 and endoglin constitutively. Stimulation with VEGF results in cell surface DAF expression, and stimulation with TNF α induces ICAM-1 and VCAM-1 expression. This indicates that the HUVEC isolated do retain the characteristics of vascular endothelium.

In the second part of the chapter, cytoprotective responses in HUVEC exposed to VEGF in the presence of LSS were investigated, exploring the hypothesis that LSS would condition EC such that a synergistic induction of HO-1 would be stimulated. I was not able to confirm this: induction of HO-1 by VEGF in static conditions was not observed, although induction by LSS was, and although a synergistic induction of HO-1 mRNA by VEGF and LSS was observed at an early time point, this was not sustained at later points and nor was it replicated with HO-1 protein expression. I also investigated changes in expression of other genes known to be affected by VEGF or LSS, but did not consistently observe a synergistic response from these either. Finally, I performed a phosphokinase array on HUVEC conditioned to LSS, then stimulated with VEGF. Although the majority of kinases included were phosphorylated either by LSS or by VEGF in static conditions, in most cases the addition of VEGF under static conditions reduced the extent of phosphorylation.

4.3.1 Induction of HO-1 by VEGF

Although three groups have previously demonstrated VEGF induction of HO-1 in EC[405-407], this was not demonstrated in my work. This may partly be because of differences in the cell conditions studied. While I studied the responses to VEGF of confluent HUVEC, analogous to the endothelium lining mature blood vessels, all three previously published studies were done in the context of angiogenesis. It is known that EC respond differently to VEGF depending on the degree of confluence and cell-cell contact maintained[164]. Part of the explanation therefore may be that the induction of HO-1 by VEGF is lessened in confluent cells.

HMOX-1 promoter polymorphisms may also be important. The *HMOX-1* promoter contains a sequence of GT repeats where the number is distributed in a bimodal fashion such that a shorter number of repeats is associated with a more readily inducible gene[413]. Given that I studied multiple isolates of HUVEC, it is possible therefore that they were derived from people with less inducible HO-1 genes. Lastly, EC isolated from some umbilical cords had high basal HO-1 expression and these tended not to show a good additional induction.

4.3.2 Effect of VEGF on HUVEC pre-conditioned with LSS

Cytoprotective gene expression

In contrast to the lack of HO-1 induction observed with VEGF, exposure to LSS for 24h resulted in a robust induction, as has been previously reported[140, 248]. However, in HUVEC pre-conditioned with LSS for 24h, I did not observe a consistent additional induction when VEGF was added, beyond a transient effect on HO-1 mRNA after 4h stimulation, that was not replicated with HO-1 protein or with mRNA at longer time points. Moreover, when the expression pattern of other genes thought to be affected by LSS and VEGF was analysed, no synergy was seen with these either. For VEGFR2 and eNOS, there was either no effect on mRNA expression or a down-regulation following VEGF stimulation after LSS pre-conditioning. The induction of DAF by VEGF was not altered by exposure to LSS, and MnSOD did not respond to LSS at all. The only gene whose expression did appear to be influenced by VEGF and LSS together was the anti-apoptotic protein A1. A striking down-regulation was observed after exposure to LSS for 28h, which was then overcome by the addition of VEGF. However, this is likely to be a transient phenomenon related to HUVEC equilibration to flow, as levels had recovered after 40h.

In addition to the lack of synergistic response to VEGF and LSS together, the changes in gene expression in those genes that responded to both (eNOS, HO-1, VEGFR2, A1) were highly variable, meaning that the results obtained are difficult to interpret with any confidence. Natural population variability in responses may be part of the explanation. However, I encountered significant technical difficulties with the Cytodyne streamer system used to expose HUVEC to shear stress. The apparatus was hard to assemble without any loss of cells, leading to variation in levels of confluence of cells as well as frequent cell loss due to an imperfect vacuum seal.

In addition, I think pre-conditioning to LSS of 16h was in hindsight too short a period and did not allow the cells to accommodate completely to flow, leading to odd transient changes in gene expression that do not reflect what would be found in arterial EC *in vivo*. This reflects wider difficulties modelling EC behaviour when exposed to flow *in vitro*. For example, when endothelial HO-1 expression is examined *in vivo* by *en face* confocal microscopy, expression levels in atheroprotected areas are very low, the opposite of that seen in *in vitro*[248]. A different flow system made by Ibidi has now been set up in Prof Mason's laboratory. This is much more user-friendly, and I would use this if I were to explore this area further in future.

Phosphokinase array

The final experiments presented in this chapter were the results of a phosphokinase array carried out to investigate changes in signalling in EC stimulated with VEGF under static conditions or conditioned by LSS. Although the results should be subject to the same caution in interpretation as those of changes in gene expression discussed in the previous section, care was taken to minimise the

potential sources of inaccuracies. Pooled HUVEC were used to reduce bias arising from using small numbers of isolates. HUVEC were exposed to LSS for 24h before the addition of VEGF, rather than 16h. Longer would have been even better, but I ran the risk of unacceptably high levels of cell loss with prolonged use of the flow apparatus. Lastly, the experiment was run three times and only samples from slides that remained confluent throughout were used in the array to prevent variations in response to VEGF being due to alterations in confluence.

The predominant pattern observed with the majority of the phosphokinases included in the array was of increased phosphorylation following exposure to VEGF or LSS compared to static untreated conditions, but a reduction in phosphorylation when VEGF was added to HUVEC conditioned to LSS. This could represent a homeostatic response, in that kinases which were perhaps optimally phosphorylated with LSS alone are then not activated further by VEGF. It may also reflect the fact that, although both LSS and VEGF are capable of inducing expression of cytoprotective genes, in other areas of EC biology they have opposing actions. For example, VEGF is associated with increased EC permeability and stimulates EC growth, migration and proliferation, while LSS reduces proliferation[156, 164].

The kinases whose phosphorylation status was enhanced by VEGF stimulation under LSS were JNK, GSK-3 α/β , β -catenin, Akt and Hck. Those that were phosphorylated equally by VEGF whether HUVEC were exposed to LSS or not were AMPK α 1, p27, ERK1/2, Hsp27 and p38 α . Activation of these kinases share several broad common functions, including cell proliferation, motility and enhancement of EC permeability. Meanwhile, p27 and AMPK α 1 inhibit cell proliferation[313, 412].

Overall there seems little evidence to support the idea of synergy between VEGF and LSS in endothelial cytoprotection, or that LSS primes EC to respond more exuberantly to VEGF as it does with other protective mediators such as statins, although technical limitations mean that important effects may have been missed. Indeed VEGF seems to act to switch off LSS-induced signalling pathways in the main and this may indicate a stabilising homeostatic influence of VEGF on the adult vasculature. However, AMPK α 1, activation of which is associated with cytoprotective effects in EC, was phosphorylated by both LSS and VEGF, although there was no additional phosphorylation seen with both together. AMPK is increasingly recognised as an important kinase in the vascular endothelium, above and beyond its well known metabolic roles. The following three chapters report findings on the pharmacological activation of AMPK and on changes in downstream cytoprotective gene expression.

5 MTX ACTIVATES AMPK AND INDUCES CYTOPROTECTIVE GENE EXPRESSION IN EC

5.1 Introduction

The second aim of the work presented in this thesis was to investigate the mechanisms underlying the protection against cardiovascular mortality seen with MTX use in RA. MTX use is associated with a reduction in CVD of 21% in the meta-analysis conducted by Micha *et al*, and up to 70% in individual studies[79, 361]. Although it is possible that this arises entirely through MTX's anti-inflammatory properties, another possibility is that MTX has effects directly on the vascular endothelium. Specifically, MTX may be operating through induction of cytoprotective pathways in EC.

It is known that the long-lasting polyglutamate metabolites of MTX alter purine and adenosine metabolism leading to increases in adenosine, which has anti-inflammatory and cytoprotective effects[369]. In addition however, inhibition of purine metabolism enzymes by MTX leads to accumulation of AMP, AICAR and ZMP, all activators of AMPK signalling. I therefore formulated the hypothesis that MTX treatment activates AMPK and induces expression of cytoprotective genes, thus enhancing endothelial resistance to injury.

5.1.1 Previous studies of MTX and AMPK

There is preliminary evidence to support the hypothesis that MTX activates AMPK. Beckers *et al* showed that MTX 5 μ M potentiated AMPK α phosphorylation by AICAR in cancer cell lines[314]. Secondly, a more recent study of a new anti-folate cancer chemotherapy agent, pemetrexed, shows that it inhibits AICAR transformylase, leading to ZMP accumulation and AMPK activation[414].

Older papers also support a role for MTX in altering cellular metabolism in ways that are consistent with activation of AMPK. High dose MTX (220 μ M) activates glycogenolysis in isolated perfused rat liver[415]. MTX also inhibited the pentose cycle enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase when used at high doses in HeLa cells[416].

If MTX does activate AMPK, this is likely then to lead to changes in expression of those genes that are targets of AMPK signalling. In EC, these include antioxidant cytoprotective genes such as MnSOD and HO-1, anti-apoptotic members of the Bcl-2 family, complement inhibitory proteins such as DAF and proteins involved in mitochondrial metabolism such as uncoupling proteins[206, 217, 276, 277, 417]. Previous studies looking at the effect of MTX on the SOD enzymes have variously shown no effect on activity or on induction in HeLa cells or in lung adenocarcinoma and mesothelioma cell lines[416, 418]. There are no studies published investigating whether low dose MTX affects expression of HO-1, Bcl-2 family members, DAF or uncoupling proteins.

5.1.2 Anti-proliferative, pro-apoptotic effects of MTX and serum concentrations

Previous studies of the effects of MTX on vascular endothelium were discussed in the general introduction. Given that MTX's predominant mechanism of action when used in cancer chemotherapy is anti-proliferative, it will be important to investigate whether it affects EC proliferation, or indeed induces apoptosis, at the concentrations achievable in patient sera when taken as a treatment for autoimmune inflammatory disease.

There are not many studies of plasma concentration of MTX. Combe *et al* measured the concentrations achieved in 20 RA patients taking a stable dose of MTX 10mg weekly (a low dose - many patients take up to 25mg). They found peak total MTX concentrations of 400nM, falling after 24h to a plateau around 10nM[419]. Similarly, Chladek *et al*, studying patients taking oral MTX 7.5 - 15mg to treat plaque psoriasis, found peak serum concentrations around 125nM, falling to a plateau between 10-20nM after 36h[420].

Previous studies suggest that MTX does exert an anti-proliferative effect on EC at doses analogous to that achieved in patient serum. Hirata *et al* found that MTX 5nM reduced DNA synthesis in HUVEC , and 0.7mg/kg weekly for 8 weeks prevented neo-vascularisation in a rabbit cornea embedded with an ECGF pellet[385]. Similarly, Yamasaki *et al* noted inhibition of HUVEC proliferation with MTX 100nM and 1000nM, without evidence of cytotoxicity[382]. However, other studies have not found a reduction in DNA synthesis with similar MTX concentrations used on the endothelial cell line EA.hy926 cells and on bovine EC[383].

No previous studies have found that MTX induces apoptosis in benign, quiescent EC grown in culture at the sort of doses found in patient sera. However , it has been found to induce apoptosis in keratinocytes at similar concentrations *in vitro*[384], and in activated T lymphocyte and monocyte cell lines[380, 421]. This last is thought to rely on generation of ROS, and blocking ROS production using NAC reduces MTX-induced apoptosis in Jurkat T cells and U937 monocytes[380].

5.1.3 Other features pertinent to studying MTX treatment of EC

MTX affects the redox balance within cells, with many studies attributing MTX toxicity, and possibly part of its therapeutic effect, to increased oxidative stress[380, 416, 422]. It therefore follows that cytoprotective responses induced by MTX might occur in response to oxidative stress as the cell attempts to restore homeostasis. In this case, one might expect a broad range of antioxidant genes to be activated by MTX treatment, including those containing anti-oxidant response elements in their promoters that are Nrf-2 transcription targets. Previous studies looking at the effect of MTX on redox-sensitive genes in HeLa cells have shown that high dose MTX inhibits glutathione reductase and γ -glutamylcysteine synthase, and depletes the pool of intracellular glutathione, but does not alter activity of SOD or catalase[416].

MTX in clinical practice is nearly always given with high dose folic acid as this is an effective way of ameliorating common gastro-intestinal side effects such as stomatitis, mouth ulcers and nausea. It would therefore be important to investigate whether the effects of MTX on EC are maintained in the presence of folic acid supplementation. Plasma concentrations in people treated with folic acid can reach up to 30 - 40nM, in patients supplemented with 2mg folic acid daily[423].

Similarly, MTX is used to treat people suffering chronic systemic inflammation, and thus it is important to model endothelial dysfunction if at all possible, by measuring whether EC maintain responsiveness in MTX after pre-treatment with pro-inflammatory cytokines sufficient to induce EC activation.

Finally, it will be important to establish whether MTX has any beneficial functional effect on EC, following on from the induction of changes in gene expression. AMPK activation in EC has previously been associated with protection against apoptosis induced by oxidative challenge and also by oxygen and glucose deprivation[262, 277]. If MTX does activate AMPK, it may therefore be capable of enhancing EC survival when exposed to glucose deprivation.

5.1.4 Chapter Aims

The experiments described in this chapter were performed to investigate the mechanism underlying the cardioprotective effect of MTX observed clinically; specifically, whether it is capable of activating AMPK and causing downstream changes in cytoprotective gene expression. The following experimental aims were addressed:

- Is MTX primarily a toxic drug that reduces EC proliferation or even induces apoptosis?
- Does MTX treatment of EC activate AMPK?
- Does MTX treatment of EC result in changes in cytoprotective gene expression?
- Does concurrent treatment of EC with folic acid change EC responses to MTX?
- Does pre-treatment of EC with pro-inflammatory cytokines alter EC response to MTX?
- Can MTX inhibit EC activation by pro-inflammatory cytokines?
- Can MTX protect EC against apoptosis?

5.2 Results

5.2.1 Effect of MTX on EC number

As MTX is known to inhibit folate metabolism, a major part of its cancer chemotherapeutic efficacy and, it was once thought, of its disease-modifying properties, is its ability to inhibit cell growth and, in malignant cells, to induce apoptosis. It was therefore hypothesised that MTX is likely to inhibit HUVEC growth in culture. An MTS assay (Promega) was used to measure EC number after treatment with MTX or vehicle control (a volume of DMSO equal to that of the highest concentration of MTX) at varying concentrations for 48h (Figure 5.1). A trend towards reduction in cell number with increasing concentrations of MTX was observed, as expected. However, the MTS assay measures viable EC numbers, and does not distinguish between increased cell death or reduced cell proliferation. Therefore, cell cycle analysis by propidium iodide staining was also performed in order to clarify whether MTX merely prevented EC proliferation or whether it induced apoptosis.

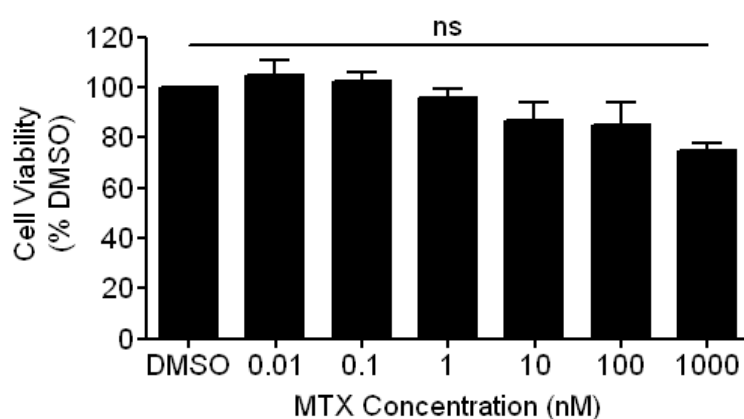


Figure 5.1 MTX reduces viable EC number. HUVEC were treated with MTX at the concentrations shown for 48h, or with DMSO vehicle control. Viable EC number was determined by loading with MTS dye and measuring absorbance at 490nm. Data shown are the results of 4 experiments. Ns: not statistically significant.

5.2.2 MTX reduces EC replication but does not induce apoptosis

Propidium iodide staining allows identification of the numbers of cells at each point in the cell cycle, as the degree of staining correlates with the amount of DNA contained within the cell. HUVEC were treated with MTX or an equal volume of DMSO for 48h at increasing concentrations, or subjected to serum starvation for 48h before cell cycle analysis. Increasing concentrations of MTX did not change the sub-G₀% (Figure 5.2), indicative of numbers of apoptotic cells, but did cause an increase in the numbers in S-phase. This suggests that MTX treatment reduces EC proliferation by preventing entry into G₂/M phase, but does not cause EC apoptosis. Although this has not been studied in HUVEC previously, this is consistent with the available literature investigating MTX effects on the cell cycle in other cell types.

Taken together with the results of the MTS assay, these results suggest that MTX reduces EC proliferation but does not precipitate apoptosis in healthy EC monolayers.

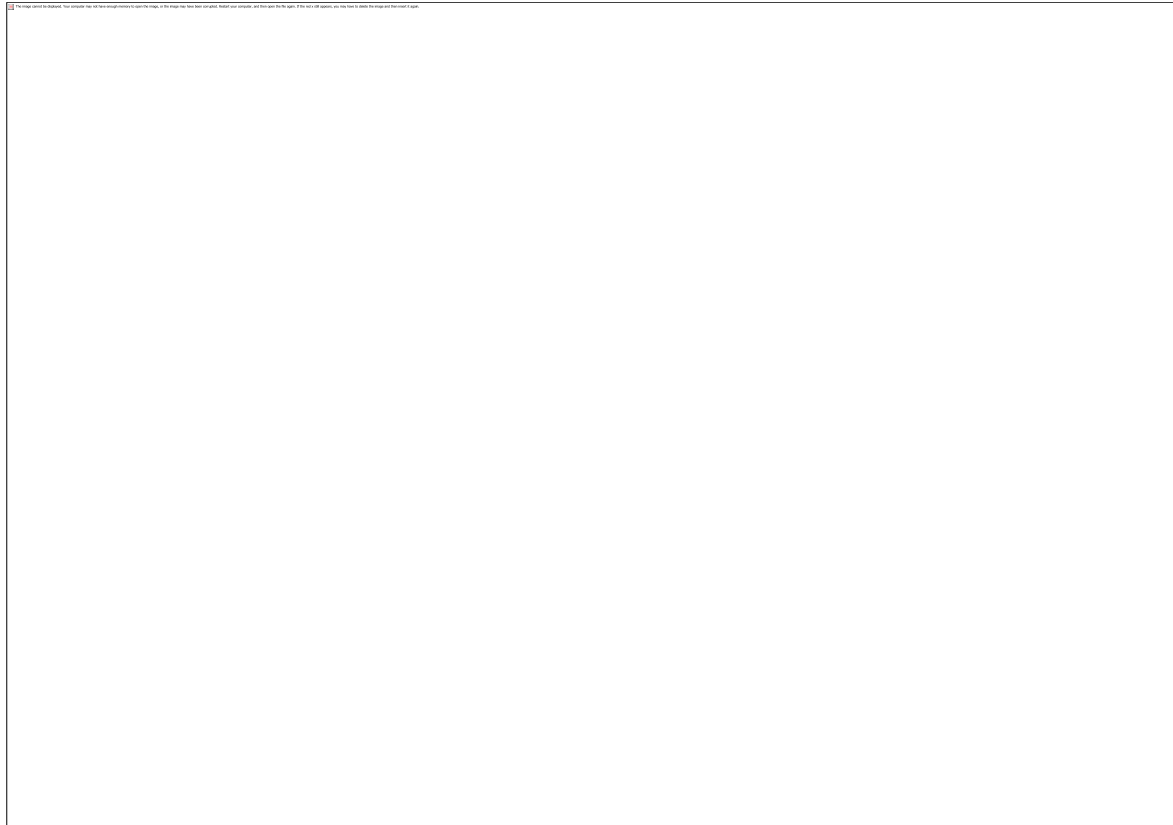


Figure 5.2 MTX reduces HUVEC replication but does not induce apoptosis. HUVEC were treated with MTX 1-100nM or vehicle control or serum starvation for 48h, then fixed and stained with propidium iodide before reading by FACS. A: typical cell cycle profile of untreated cells; B: typical cell cycle profile of cells treated with MTX 100nM for 48h; C: percentage of apoptotic (sub G₀) cells; D: percentage of EC in S-phase. C and D represent the results of 6 experiments, expressed as mean $\pm$ standard error. UT: vehicle control; SS: serum starvation for 48h; ns: not statistically significant; ** = $p < 0.01$; *** = $p < 0.001$.

5.2.3 MTX treatment phosphorylates AMPK α in HUVEC

Given that the currently accepted mechanism of action of MTX involves intracellular accumulation of AICAR and AMP, and that these are known activators of AMPK, it was hypothesised that MTX may activate AMPK. HUVEC were treated with MTX 100nM, vehicle control or AICAR 1mM as a positive control for 24-72h. The concentration chosen was based on that able to increase intracellular AICAR[375] and to be measured in patient sera following therapeutic dosing[419]. Because of technical difficulties with stripping and reprobing a single membrane, each condition was loaded onto two gels, and phosphorylated AMPK and total AMPK were blotted for separately, and corrected for loading by probing each membrane separately for GAPDH.

A two-fold increase in AMPK α^{Thr172} phosphorylation was observed at 48h, but not at 24h (Figure 5.3A and B), consistent with this being a delayed response dependent on the production of MTX polyglutamate metabolites, and the accumulation of AICAR and AMP following enzyme inhibition. A less consistent increase in phosphorylation was observed at 72h (Figure 5.3C). It should also be noted that there was a degree of variation in basal level of AMPK phosphorylation.

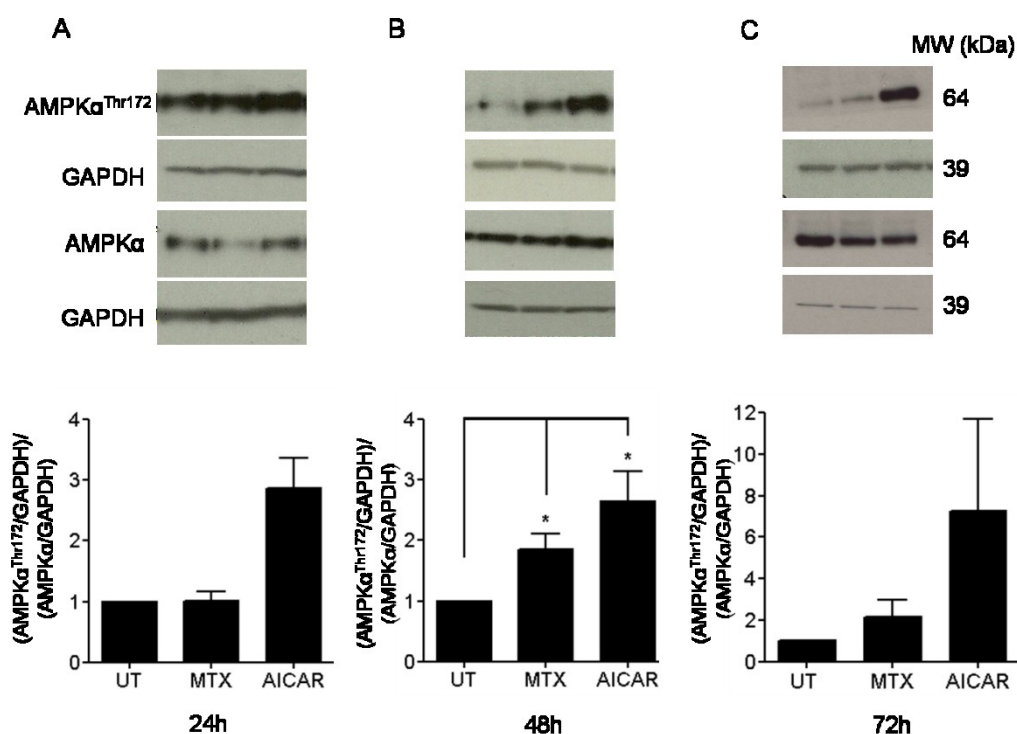


Figure 5.3 MTX phosphorylates AMPK α in HUVEC. HUVEC were treated with MTX 100nM or AICAR 1mM as positive control for 24h (A), 48h (B) or 72h (C). Representative blots and densitometry are shown, with AMPK α^{Thr172} expressed relative to total AMPK α and GAPDH. Data plotted are mean $\pm$ SEM and represent the results of 3-6 experiments. UT = vehicle control; *= $p < 0.05$.

5.2.4 MTX induces MnSOD expression in HUVEC and HAEC

Following the discovery that MTX can activate AMPK in EC, I explored whether MTX treatment results in induction of cytoprotective genes known to be targets of AMPK signalling. MnSOD is an antioxidant enzyme, essential for life, that catalyses the conversion of superoxide radicals into H_2O_2 , and is located in mitochondria[221]. Induction of MnSOD is an important vascular cytoprotective response, capable of protecting against mitochondrial damage and atherogenesis[226].

Induction of MnSOD mRNA and protein was seen after 48h MTX treatment in HUVEC, and maintained at 72h (Figure 5.4A and C). This response was concentration-dependent, first seen with MTX 0.1nM and maximal at 10 and 100nM (Figure 5.4D). Moreover, a two-fold induction in MnSOD protein was reproduced in HAEC treated with MTX 100nM for 48h (Figure 5.4B). Again, consistent with this being linked to AMPK activation and dependent on changes in purine nucleotide levels, the induction was only observed after 48h treatment and not at earlier time points. Increased MnSOD expression was also shown following AICAR treatment of HUVEC (Figure 6.4) , and has previously been linked with AMPK activity[262, 276, 310].

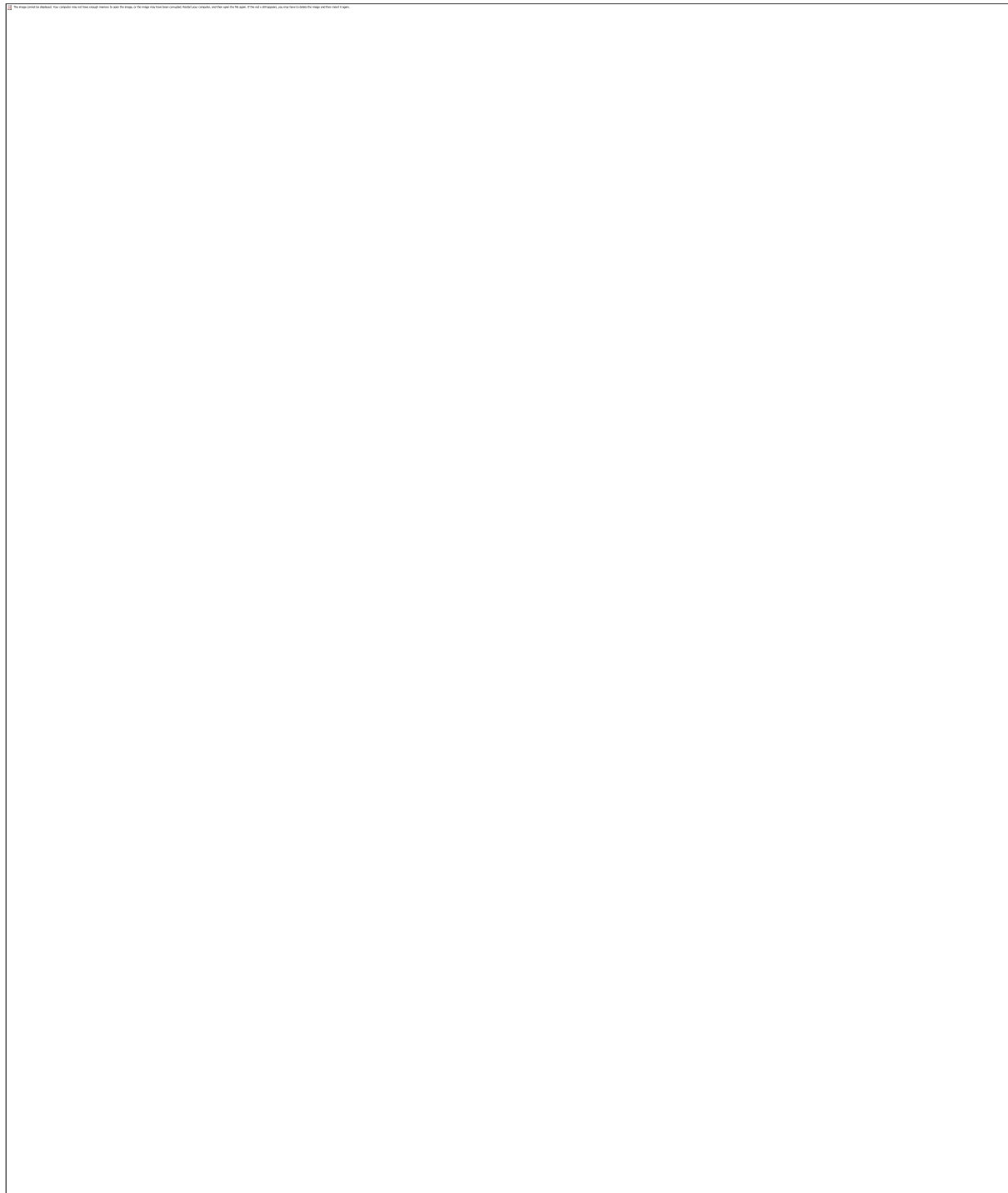


Figure 5.4 MTX induces MnSOD mRNA and protein. HUVEC were treated with MTX at concentrations varying from 0.1nM to 100nM for 24-72h. A: representative western blot and corresponding densitometry showing MnSOD protein expression in HUVEC in response to increasing MTX concentrations for 48h. B: representative western blot and corresponding densitometry demonstrating MnSOD induction by MTX 100nM for 48h in HAEC. C: time course of MnSOD mRNA induction in HUVEC following stimulation with MTX 100nM. D: dose response of MnSOD mRNA induction after stimulation with MTX for 48h. Data shown are mean $\pm$ SE and are the results of >3 experiments. UT = vehicle control; *= $p<0.05$; **= $p<0.01$.

5.2.5 MTX induces HO-1 expression in HUVEC

Another known AMPK target in EC is the anti-oxidant, anti-inflammatory and anti-apoptotic enzyme HO-1. HO-1 is an extremely inducible enzyme which catalyses the breakdown of heme into bilirubin, creating carbon monoxide, iron and biliverdin as its end-products. Its induction in EC is associated with vascular health in a number of different ways, including resistance to transplant vasculopathy. Treatment of EC with MTX resulted in a dose dependent induction of HO-1 mRNA and protein, most pronounced at 10 and 100nM, and after 48h treatment (Figure 5.5).

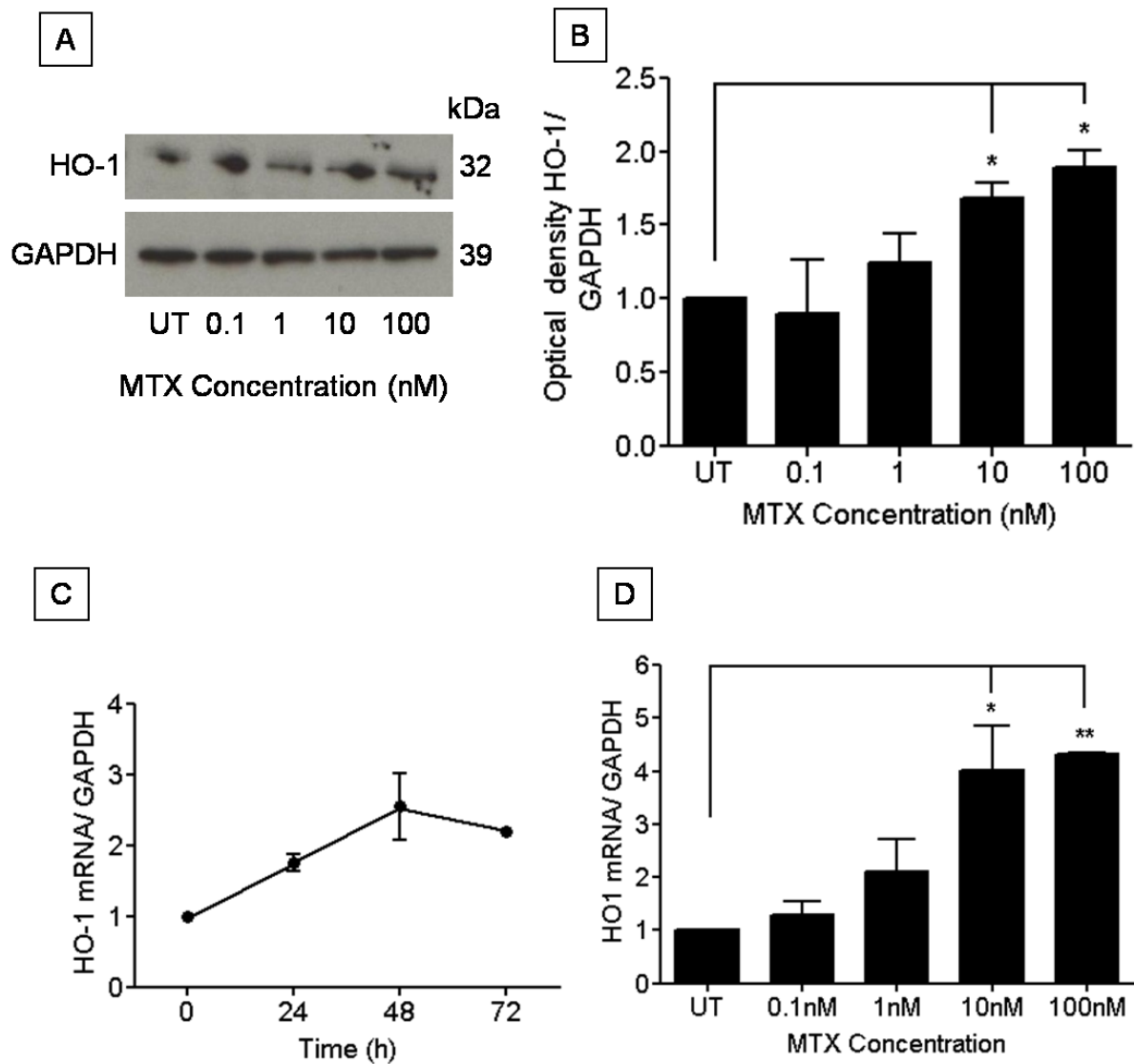


Figure 5.5 MTX treatment induces HO-1 mRNA and protein expression. HUVEC were treated with MTX 0.1 – 10nM for 0 to 72h. A: representative western blot showing HO-1 protein expression in response to rising concentrations of MTX for 48h. B: corresponding densitometry. C: Time course of HO-1 mRNA induction following stimulation with MTX 100nM for 24-72h. D: HO-1 mRNA induction in response to increasing concentrations of MTX over 48h. Data shown represent the results of >3 experiments. UT = vehicle control; *= $p < 0.05$; **= $p < 0.01$.

5.2.6 MTX induces expression of other AMPK target genes

AMPK signalling results in the induction of many target genes with cytoprotective functions: antioxidants, anti-apoptotic proteins, proteins involved in the regulation of mitochondrial ROS production and promoting oxidative phosphorylation, and anti-inflammatory genes. mRNA from HUVEC treated with MTX 100nM for 48h was therefore analysed to look for changes in other cytoprotective genes known to be a target of AMPK.

The Bcl-2 family of proteins have both pro- and anti-apoptotic members. A 12-fold induction of A1 mRNA, an anti-apoptotic member of the family, was observed (Figure 5.6A). Induction of this gene has not previously been linked to MTX use or to AMPK signalling. A trend towards induction of Bcl-2 itself was observed (Figure 5.6B) but this was not statistically significant.

Induction of UCP2 mRNA was also seen in HUVEC treated with MTX after 48h (Figure 5.6C). UCP-2 is involved in the regulation of mitochondrial ROS production, by dissipating the proton gradient that drives ATP generation. It has also recently been implicated in the maintenance of endothelial homeostasis[241].

Lastly, in HUVEC treated with MTX for 72h, induction of DAF was observed (Figure 5.6D). This complement-inhibitory protein prevents the formation and accelerates the decay of C3 and C5 convertases, minimizing deposition of C3 and generation of C3a, C5a and C5b-9[237]. Thus, MTX-mediated AMPK activation in EC is linked to the regulation of a variety of AMPK-dependent protective genes, all of which are anti-atherogenic.

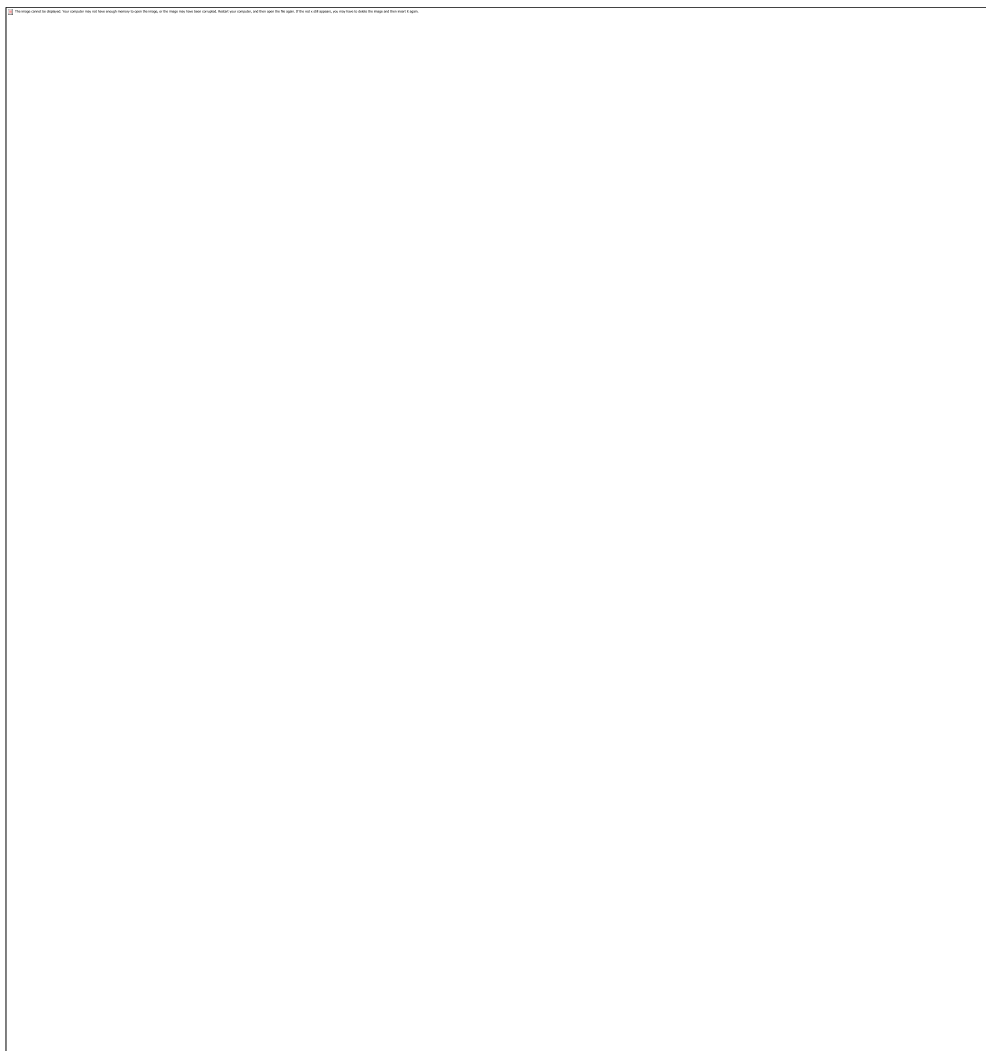


Figure 5.6 MTX treatment causes induction of other AMPK target genes. HUVEC were treated with MTX 100nM for 48h (A-C) or 72h (D). Relative mRNA levels were determined by qRT-PCR, using β -actin as housekeeping gene. A: induction of A1; B: induction of Bcl-2; C: induction of UCP-2 and D: induction of DAF. Data shown are the results of 4 experiments. UT = vehicle control; *= $p<0.05$; **= $p<0.01$.

5.2.7 MTX does not cause a general induction of antioxidant enzymes

Given that it has been demonstrated that MTX induces expression of two anti-oxidant enzymes in HUVEC (MnSOD and HO-1), induction of other anti-oxidant enzymes was investigated. mRNA from HUVEC treated with MTX 100nM for 48h was analysed by qRTPCR to look at relative expression levels of NQO-1, Trx, and Trx reductase. No induction was seen (Figure 5.7). These were the same samples in which induction of MnSOD, HO-1 and other AMPK target genes was observed, indicating that the cells had responded to MTX and validating this result. Thus, MTX does not appear to elicit a general rise in anti-oxidant enzymes, but rather promotes the specific induction of certain genes.

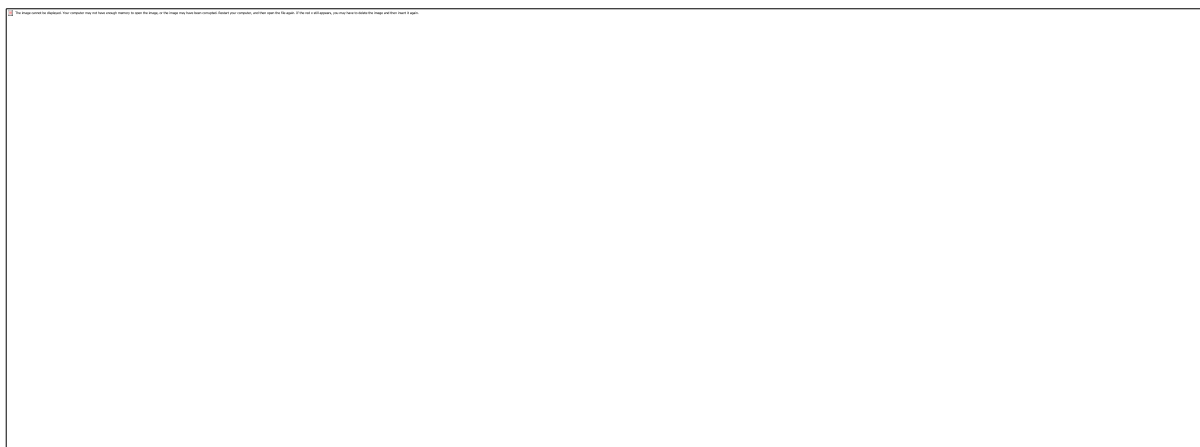


Figure 5.7 MTX does not induce expression of other antioxidant genes. HUVEC were treated with MTX 100nM for 48h, and mRNA levels of the antioxidant genes NQO-1 (A), thioredoxin (B) and thioredoxin reductase (C) were measured. Data shown represent the results of 4 experiments. UT = vehicle control.

5.2.8 Co-treatment with MTX and folic acid does not alter MnSOD induction

In an attempt to determine whether the MTX-induced changes in gene expression in quiescent EC are relevant to the clinical scenario, responses in EC co-treated with folic acid (FA) were investigated. FA is routinely prescribed alongside MTX to mitigate side effects and it was therefore important to establish whether the observed changes in gene expression were maintained in its presence. In the first experiments, EC were treated with MTX 100nM for 48h, and FA 50nM for the second 24h. The dose was chosen to reflect the plasma concentrations achieved in people given FA supplementation[423-425]. No influence of FA on induction of MnSOD mRNA was observed (Figure 5.8A). Secondly, HUVEC were treated with MTX 100nM for 48h, or a 10-fold dilution series of FA from 50nM to 50μM for 24h. FA even at 50μM did not alter HO-1 or MnSOD mRNA levels, and there was no evidence of a dose response in cells treated with MTX and FA together. It should be noted that this latter experiment was performed only once (Figure 5.8B and C). However, as it showed no difference in HO-1 induction with folic acid added, this was not therefore repeated on the samples used to generate Figure 5.8.

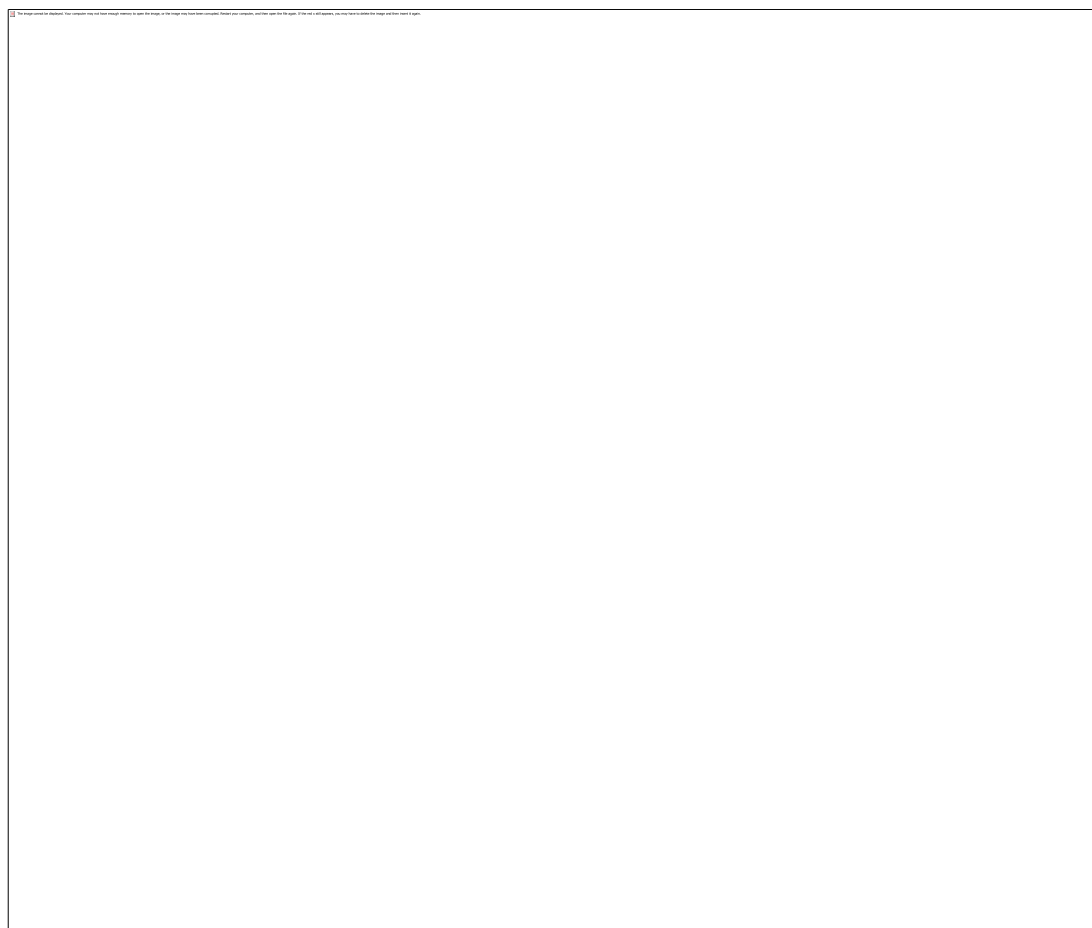


Figure 5.8 Co-treatment of EC with folic acid does not alter the induction of MnSOD mRNA by MTX. A: HUVEC were treated with MTX 10nM for 48h and FA 50nM for 24h. MnSOD mRNA induction was measured by qRT-PCR. Data shown are the results of 4 experiments. B and C: HUVEC were treated with MTX 100nM

for 48h and FA in varying concentrations from 0.05 μ M to 50 μ M for 24h, before analysis of MnSOD and HO-1 mRNA levels by qRT-PCR. Data shown are the results of 1 experiment. UT: vehicle control; FA: folic acid; ns: not statistically significant; *=p<0.05.

5.2.9 EC pre-treated with TNF α maintain responsiveness to MTX

Patients with RA and those with primary coronary artery disease exhibit endothelial dysfunction as an early feature. This is characterised by reduced NO bioavailability, often linked to excess ROS production, increased endothelial apoptosis and lipid permeability, and impaired endothelial responses to exogenous stimuli. The pro-inflammatory cytokine TNF α is an important mediator of endothelial dysfunction and was chosen to model this *in vitro*. EC were exposed to TNF α (1ng/ml) for 24h prior to the addition of MTX for 48h. MTX-induced upregulation of MnSOD and HO-1 mRNA was preserved, and as expected a small induction of MnSOD with TNF α alone was also seen (Figure 5.9A and B). Overall, the effect of TNF α and MTX together on MnSOD induction appeared to be additive.

To characterise further the induction of MnSOD mRNA by MTX and TNF α , a time course was performed (Figure 5.9C). This was not done to look at HO-1 mRNA induction as this is not known to be a target of TNF α , unlike MnSOD, and no induction was observed with TNF α alone in the preceding experiments. The time course showed that TNF α causes a rapid induction of MnSOD that peaks at 4-8h and has largely subsided by 24h. By contrast, MTX causes a delayed induction, maximal at 48h. This may have implications for the mechanisms by which TNF α and MTX induce MnSOD: while TNF α causes a direct activation of NF κ B which alters gene expression in a matter of hours, MTX is likely to be effecting changes indirectly through alterations in various purine pathway metabolites. Alterations in gene expression are delayed while levels of these metabolites build up.

These findings confirm the ability of MTX to condition EC in the face of a chronic pro-inflammatory stimulus such as that seen in patients with RA.

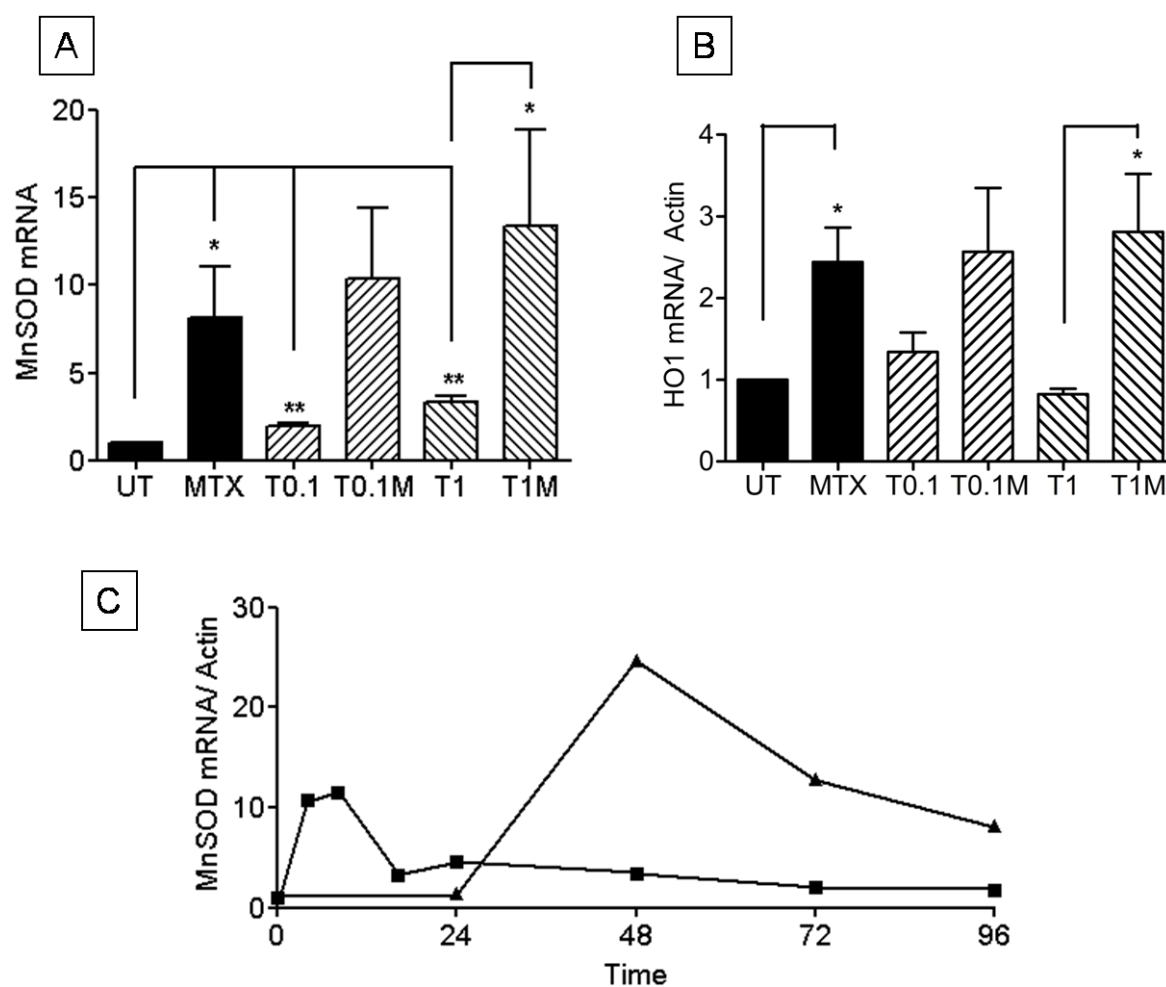


Figure 5.9 Induction of MnSOD and HO-1 by MTX still occurs when HUVEC are pre-treated with TNF α . A and B: HUVEC were treated with TNF α 0.1ng/ml or 1 ng/ml for 24h, before the addition of MTX 10nM for 48h. MnSOD and HO-1 mRNA levels were measured by qRT-PCR. Data represent the results of 5 experiments. UT: vehicle control; T0.1: TNF 0.1ng/ml; T0.1M: TNF 0.2ng/ml + MTX 10nM; T1: TNF 1ng/ml; T1M: TNF 1ng/ml + MTX 10nM. *= $p < 0.05$; **= $p < 0.01$. C: Time course of MnSOD mRNA induction by TNF 1ng/ml and MTX 10nM. Data represents the results of 1 experiment. Black squares: TNF treatment; black triangles: MTX treatment.

5.2.10 MTX does not alter ICAM-1 and VCAM-1 induction by TNF α *in vitro*

In order to explore further how EC pre-treated with TNF α respond to MTX, ICAM-1 and VCAM-1 cell-surface expression was investigated by flow cytometry. These are canonical TNF α target genes, responsible for EC-leucocyte adhesion. HUVEC were treated with MTX 10nM for 36h, before the addition of TNF α 1 or 10ng/ml for 16h. As expected, a brisk induction of both VCAM-1 and ICAM-1 with TNF α was observed. However, this was not reduced by the simultaneous addition of MTX, and indeed MTX alone caused a small induction of both VCAM-1 and ICAM-1 on the cell surface (Figure 5.10).

These findings were unexpected, given that MTX is an anti-inflammatory drug and previous studies in HUVEC and in samples from patients have suggested that it reduces ICAM-1 and VCAM-1 expression[382]. They were further investigated by looking at a time course of changes in ICAM-1 mRNA and both ICAM-1 and VCAM-1 cell-surface expression in response to TNF α and MTX (Figure 5.11). These indicated that the magnitude of induction seen with MTX was many times smaller than that seen with TNF α . These results may reflect a non-specific effect of MTX on cells in culture as they do not correspond to the situation seen in patients treated with MTX. They highlight however the limits to which conclusions about drug actions can be drawn from *in vitro* experiments only.



Figure 5.10 MTX pre-treatment does not reduce induction of ICAM-1 or VCAM-1 by TNF α . HUVEC pre-treated with MTX 10nM for 36h were stimulated with TNF α 1 and 10ng/ml for 16h. ICAM-1 and VCAM-1 expression were analysed by FACS. A and C: representative uncompensated FACS images of ICAM-1 and VCAM-1 expression respectively. Yellow: untreated; red: MTX 10nM; blue: TNF α 10ng/ml; green: MTX and TNF α . B and D: normalised RFI for ICAM-1 and VCAM-1 respectively. UT: vehicle control; T1: TNF α 1ng/ml; T10 TNF α 10ng/ml; MT1: MTX 10nM and TNF α 1ng/ml; MT10: MTX 10nM and TNF α 10ng/ml. Data are the results of 3 experiments. * = $p < 0.05$; ** = $p < 0.01$.

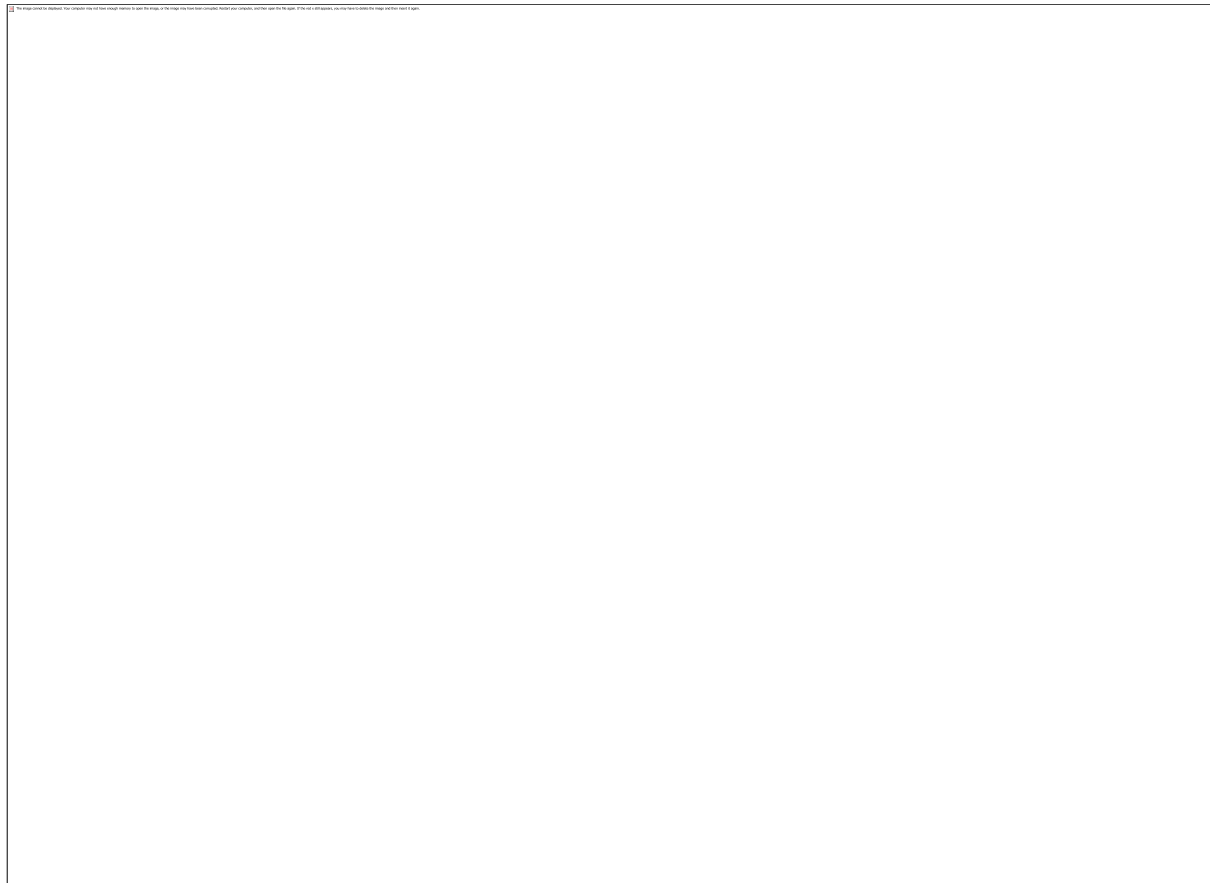


Figure 5.11 Time course of ICAM-1 and VCAM-1 induction by TNF α and MTX. HUVEC were stimulated with TNF α 1ng/ml or MTX 10nM for varying times. Untreated cells were also analysed and the results subtracted from those of the stimulated cells. A: time course of ICAM-1 mRNA induction; B: time course of ICAM-1 cell surface expression; C: time course of VCAM-1 cell surface expression. Squares: TNF α ; triangles: MTX. Data shown are the results of one experiment.

5.2.11 MTX protects against apoptosis induced by glucose deprivation

AMPK is principally known for its role in conserving cellular energy[181]. Thus, in order to determine whether its activation by MTX was functionally important in EC, a glucose deprivation assay was used. Similar assays have been used in previous studies of AMPK-mediated protection against apoptosis, sometimes in combination with oxygen deprivation. EC were treated with MTX 100nM for 48h, and then maintained for 18h in HBSS or glucose-deficient HBSS. Cell viability was initially determined by Trypan blue exclusion, and 18h glucose deprivation resulted in a marked increase in numbers of dead EC (Figure 5.12) while MTX alone made no difference to the numbers of dead cells, as expected from the results of early experiments with MTS and cell cycle analysis (Figures 5.1 and 5.2).

In order to study death by apoptosis specifically, early apoptosis was detected by staining with Annexin V which binds phosphatidylserine, the membrane phospholipid exposed when the inner surface of the cell membrane is flipped outwards early in the process. Established cell death was detected by permeability to propidium iodide, using flow-cytometric quantification. Glucose deprivation led to a marked increase in binding of Annexin V to the EC surface and resulted in a doubling of PI-positive cells. These responses were significantly reduced by MTX (Figure 5.13). Moreover, MTX alone did not increase EC apoptosis under normal glucose conditions. These data suggest that the effect of MTX treatment on vascular EC is protective, as opposed to representing a stress response to a noxious stimulus.



Figure 5.12 Glucose deprivation induces apoptosis. HUVEC were treated with MTX 100nM for 54h, then incubated either in HBSS containing 2% BSA and L-glutamine, or modified HBSS without glucose for 18h. A: photomicrograph of HUVEC in HBSS; B: photomicrograph of HUVEC pre-treated with MTX in HBSS; C: photomicrograph of HUVEC after undergoing glucose deprivation for 18h; D: photomicrograph of HUVEC pre-treated with MTX then deprived of glucose for 18h. All taken at x40 magnification. E: analysis of trypan blue staining of HUVEC. HBSS: HUVEC incubated in HBSS; HBSS M: pre-treated with MTX then incubated in HBSS; GD: HUVEC incubated in HBSS without glucose; GD M: HUVEC pre-treated with MTX then incubated in HBSS without glucose. *** = $p < 0.001$.



Figure 5.13 MTX protects EC from apoptosis induced by glucose deprivation. HUVEC were pre-treated with MTX 100nM for 54h, before glucose deprivation for 18h. Early apoptosis was detected by Annexin V staining (A and C), and cell death by propidium iodide positivity (B and D). A: representative FACS image depicting Annexin V staining. Green: untreated cells in HBSS; red: glucose deprivation; blue: glucose deprivation after MTX pre-treatment. B: representative FACS image depicting propidium iodide positivity. Black: untreated cells in HBSS; red: glucose deprivation; blue: glucose deprivation in cells pre-treated with MTX. C: normalised relative fluorescence intensity of Annexin V staining, representing 3 experiments. D: percentage propidium iodide positivity, representing 6 experiments. APC: allophycocyanin; PE-Cy5: Phycoerythrin-cyanine 5; HBSS: HUVEC incubated in HBSS with 2% BSA and L-glutamine; HBSS M: HUVEC pre-treated with MTX then incubated in HBSS as before; GD: HUVEC incubated in HBSS without glucose, with 2% BSA and L-glutamine; GD M: HUVEC pretreated with MTX incubated in HBSS without glucose as before. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

5.3 Discussion

The results presented in this chapter demonstrate that low-dose MTX exerts changes in signalling and protective gene expression in HUVEC in culture. Specifically, low-dose MTX activates AMPK and induces MnSOD, HO-1 and other known AMPK target genes including UCP2, DAF and A1.

Moreover these changes do not involve a general rise in anti-oxidant enzymes, perhaps suggesting that they represent a specific signalling response and not a general defence against oxidative stress.

The effects are preserved when EC are pre-treated with FA, and also when EC are exposed to TNF α to mimic a dysfunctional endothelium. Moreover, although MTX reduces EC proliferation, it does not induce apoptosis, and indeed was able to provide some protection against apoptosis induced by glucose deprivation. However, it did not alter induction of cell adhesion molecules by TNF α .

5.3.1 Functional effects of MTX on EC viability and apoptosis

One major concern with the results presented in this chapter is whether the observed changes in protective gene expression are occurring because MTX is a noxious stimulus, causing oxidative stress or even inducing apoptosis in EC, as it does in activated leucocytes. Some support to this notion was provided by the observation that MTX reduces cell proliferation by inducing a cell cycle block at the S-phase - G2/M phase transition. A cell cycle block at this stage with MTX has previously been reported in colon cancer cell lines, activated T lymphocytes and in astrocytes in culture, and in these papers was associated with increased apoptosis[426-428]. That this is not the case was shown by experiments indicating that MTX does not increase the sub G₀% in cell cycle analysis, and does not induce apoptosis measured either by trypan blue staining, Annexin V binding or propidium iodide permeability. In fact, MTX protected EC against apoptosis when induced by glucose deprivation.

It should be noted that in the experiments presented here, the effect of MTX on EC proliferation, as measured by MTS assay, was not statistically significant (Figure 5.1). Given the clear dose response, I interpret this as being due to small n numbers and not because of a lack of biological effect. An effect of low dose MTX on EC proliferation is, however, much less likely to be relevant *in vivo* given that EC turnover in mature vascular beds is much lower than that seen in cells in culture[87].

MTX treatment of EC exerted a demonstrable functional effect and was able to attenuate apoptosis induced by complete glucose deprivation. The mechanism underlying this response is likely multifactorial. AMPK activation moves cells away from glycolysis as an ATP source, with glucose used more efficiently by oxidative phosphorylation in the mitochondria, thus prolonging cell survival in low glucose conditions. Additionally, the induction of A1 and HO-1 by MTX may exert anti-apoptotic effects. The induction of MnSOD, HO-1 and UCP-2 will also impart important anti-oxidant effects which are likely to improve cell survival.

5.3.2 AMPK activation by MTX

A notable feature of the experiments presented here is the relatively long delay between exposing HUVEC to MTX, and the onset of changes in AMPK phosphorylation state and changes in gene expression. HUVEC were treated with MTX for 48h before AMPK α phosphorylation and MnSOD and HO-1 induction occurred, and 72h for DAF induction. This delay is consistent with activation of AMPK by MTX occurring due to changes in intracellular AICAR, ZMP and AMP levels as a result of enzyme inhibition, as I speculate that these would take some time to accumulate following MTX conversion to polyglutamate metabolites and subsequent inhibition of AICAR transformylase and adenosine deaminase. Although no experiments have been reported here that investigate this, Cronstein *et al* have previously shown that treating HUVEC with MTX 100nM for 48h results in measurable increases in intracellular AICAR concentration[375], and Beckers *et al* show that treating cancer cell lines with MTX and AICAR together increases ZMP concentrations and enhances the AMPK α phosphorylation seen with AICAR treatment alone[314]. These studies support the hypothesis that MTX activates AMPK by increasing concentrations of AICAR, leading in turn to increased concentrations of the allosteric activators of AMPK, AMP and ZMP.

The choice of MTX and FA concentration was very carefully made, based on the plasma concentrations achievable in patients treated with MTX for chronic inflammatory conditions[419, 420, 423]. This improves the chance that the changes in AMPK signalling and gene expression seen are applicable to the clinical situation, but nevertheless this is a long way from full confidence that these results are pertinent to the actions of MTX when taken by patients.

AMPK signalling is a significant mediator of vascular protection, and activation in EC by MTX, at concentrations achievable in clinical practice, is highly suggestive that this is a clinically relevant new mechanism of action for MTX. It could potentially explain why MTX improves CV mortality in RA. In EC, AMPK activation has been linked to eNOS activation and enhanced NO production, protection against apoptosis, beneficial effects on mitochondrial metabolism and on antioxidant gene expression; a small proportion of which have been investigated here[262, 276, 277, 292]. Further research would be needed to establish whether MTX is capable of replicating all effects previously ascribed to AMPK activation in EC.

If MTX activates AMPK in EC, it is possible that it also does so in other cells. AMPK signalling and the metabolic changes it promotes have been linked to anti-inflammatory processes in cells of the immune system[266]. It is possible therefore, that AMPK activation underlies the anti-inflammatory effects of MTX treatment, alongside the changes in adenosine metabolism previously discussed.

5.3.3 Induction of MnSOD by MTX

This chapter demonstrated that MTX treatment induced MnSOD expression in both HUVEC and HAEC, with significant increases in mRNA transcripts shown even at MTX concentrations of 0.1nM. MnSOD, the SOD isoform found within mitochondria, has previously been linked with vascular protection and with AMPK activation and thus its induction may be an important mechanism by which MTX preserves endothelial function[226, 310]. In addition, it is possible that MnSOD activation plays a part in the anti-inflammatory actions of MTX. Cuzzocrea *et al* showed that treating rats with collagen-induced arthritis with MTX and a SOD mimetic together allowed a 10-fold diminution in the dose of MTX needed to have a disease-altering effect[394]. *In vitro*, MTX has been shown to reduce superoxide production by neutrophils, which could involve increases in MnSOD expression or activity[429].

A further limitation is the degree to which experimental results obtained in HUVEC can be extrapolated to other vascular EC types. Although using multiple isolates of early passage HUVEC has the advantage of maintaining some population variability, clearly the umbilical vein is not a vessel ever likely to develop an atherosclerotic plaque. To overcome this, induction of MnSOD protein at least was reproduced in HAEC, and in Chapter 7 the results of MTX treatment on murine vascular MnSOD and AMPK expression are presented.

5.3.4 Induction of other cytoprotective genes by MTX

I also demonstrated in this chapter that MTX treatment increases expression of other known AMPK targets: HO-1, DAF, A1 and UCP-2. There are no pubmed-cited articles exploring a relationship between MTX and HO-1, DAF, A1 or UCP-2. However, all may be relevant to what we know of how MTX operates. Induction of HO-1 is often linked with anti-inflammatory effects in addition to being anti-oxidant, and so HO-1 expression could be an important facet of MTX mechanism of action[430].

Induction of the anti-apoptotic Bcl-2 family protein A1 by MTX seems counterintuitive given its actions in cancer chemotherapy, where MTX causes apoptosis of tumour cells. That EC behave differently demonstrates the differences between the responses of non-malignant primary cells and cancer cell lines, and potentially between the responses to different doses of MTX, with high doses being toxic but low doses protective.

Induction of UCP-2, alongside MnSOD by MTX, coupled to the previous knowledge that MTX alters ROS production in leucocytes, raises the possibility that it is altering mitochondrial metabolism and signalling.

Although MTX treatment led to induction of two anti-oxidant enzymes, MnSOD and HO-1, this was not part of a general induction of anti-oxidant mechanisms, as no induction at all was seen with Trx, Trx reductase or NQO-1. Speculatively, this suggests that MTX treatment does not affect activity of

the Nrf-2/ARE system, which is an important regulator of transcription of these enzymes. Although HO-1 expression can be regulated via Nrf-2, MnSOD is not[140].

5.3.5 MTX does not alter TNF α -stimulated cell adhesion molecule expression *in vitro*

One surprising finding was the failure of MTX to inhibit the induction by TNF α of the cell adhesion molecules ICAM-1 and VCAM-1. Moreover, expression of these was slightly increased by MTX itself. This is not consistent with previously published studies of the effects of MTX on cell adhesion molecule expression: soluble ICAM-1 and VCAM-1 are reduced in patients with early RA treated for 6 months with MTX[431]; other researchers have found a reduction in ICAM-1 and VCAM-1 in HUVEC treated with MTX for similar time periods and concentrations (although quantified by western blot and immunofluorescence rather than flow cytometry)[382]; and AMPK activation has previously been demonstrated to reduce endothelial NF κ B-mediated transcription, the major driver of ICAM-1 and VCAM-1 expression[335]. This may represent an artefact relating to the *in vitro* conditions, or may relate to the ability of MTX to alter ROS levels, as these can play a role in the TNF α -mediated induction of cell adhesion molecules. The effect of MTX on vascular ICAM-1 expression was further explored *in vivo* and the results are shown in Chapter 7, underlining the importance of not relying solely on *in vitro* findings when drawing conclusions about how biological mechanisms really operate.

6 MECHANISM OF INDUCTION OF MNSOD AND HO-1 BY MTX

6.1 Introduction

This chapter presents data exploring how MTX treatment of EC leads to induction of the cytoprotective genes MnSOD and HO-1. It has already been demonstrated that prolonged low-dose MTX leads to AMPK phosphorylation and induction of cytoprotective genes including MnSOD and HO-1; therefore, whether AMPK activation is required for this induction by MTX was studied. CREB is a phosphorylation-dependent transcription factor known to be a direct target of AMPK and also known to bind the promoters and activate transcription of MnSOD and HO-1. Therefore, its role in their induction was also explored. Finally, the role of ROS was investigated.

6.1.1 Prior studies of AMPK induction of MnSOD and HO-1

AMPK activation has been shown previously to lead to HO-1 induction in some but not all settings in EC. While Liu *et al* found that AMPK activation with AICAR did induce HO-1 in HUVEC, HAEC and rat carotid arteries, leading to protection against apoptosis, Colombo and Moncada, who used hypoxia as an AMPK activator, did not observe HO-1 induction[217, 276]. MnSOD induction following AMPK activation with AICAR or metformin has also been demonstrated in EC[262, 310].

6.1.2 CREB

CREB was the first phosphorylation-dependent transcription factor identified[432]. It is a member of a family that also includes CREM and ATF-1, the defining feature of which is that all members are capable of stimulating gene expression from promoters containing cAMP-responsive element (CRE) sites[433]. It is involved in the regulation of cell survival, proliferation, pro- and anti-inflammatory gene expression and the mediation of fasting and feeding signals on the expression of metabolic genes in insulin-sensitive tissues (ie liver, pancreas, adipose tissue and skeletal muscle)[199, 433]. A wide variety of signals can induce phosphorylation, but gene transcription is initiated in response in only a subset of genes to which it is capable of binding. How CREB discriminates between activating signals and directs transcription in a specific subset is not yet understood.

The best characterised extra-cellular signals that activate CREB are the small peptide hormones such as glucagon that bind G-protein-coupled receptors, leading to increased activity of adenylyl cyclase and rising concentrations of the archetypal second messenger cAMP[434]. However, it can also respond to signals from growth factors and from adverse stimuli such as osmotic stress and UV light[433]. The principal upstream activator of CREB is cAMP. Rising concentrations of cAMP activate PKA which is capable of direct phosphorylation of CREB at Ser133. AMPK, PKC and

CaMKII can also phosphorylate CREB on the same serine residue[434]. Dephosphorylation and thus de-activation is controlled by two serine/ threonine phosphatases: PP1 and PP2A[435].

The CREB molecule structure consists of an amino terminal transactivation domain and a carboxy terminal basic leucine zipper domain. The transactivation domain itself consists of two parts: a kinase-inducible domain (KID) and a constitutive activation domain. The KID contains the Ser133 residue that is the phosphorylation target and thus mediates the ability of CREB to activate in response to cAMP and calcium through phosphorylation[434].

CREB binds to DNA at CRE sites - a palindromic sequence TGACGTCA - or half-sites - TGACG or CGTCA[434]. These sequences occur approximately 750 000 times in the human genome, but most are unoccupied due to a cysteine methylation within the site that disrupts CREB binding[436]. Around 4000 genes are thought to have functional CRE sites: unmethylated sequences lying within 250 bp of the transcription start[436]. CREB is constitutively bound to a large proportion of these and can also be phosphorylated in a large number in response to cAMP, but only genes with TATA boxes are capable of responding to a cAMP signal with induction of transcription[437]. CREB is more active the closer it is to a TATA box. However, TATA boxes are not present in about two thirds of CREB-occupied promoters, raising a question about how gene expression is regulated by CREB in these cases[433]. Of note, the *SOD2* promoter does not contain a TATA box.

Phosphorylation on Ser133 in CREB, although not enough alone to activate gene transcription, facilitates recruitment of co-activators, of which there are at least two sorts. Firstly, CREB-binding protein (CBP) and p300 are coactivators which bind to the KID[434, 438]. They can modify histone acetylation on promoters[439], and recruit RNA polymerase II[440], and their recruitment is more predictive of increased gene expression than the promoter being occupied by phCREB in response to cAMP[436].

The second group of CREB co-activators are CREB-regulated transcription coactivators (CRTCs). These bind to the leucine-zipper DNA-binding domain. They normally reside in the cytoplasm, and are activated themselves through de-phosphorylation. Following dephosphorylation they can translocate to the nucleus and interact with CREB[433, 441]. In addition to affecting gene expression, CRTC binding may mediate some effects of CREB on alternative splicing, and these effects have been demonstrated in TATA-less genes, which may provide a partial explanation for how CREB affects gene expression in this group[433]. It is through regulation of the binding of the coactivators that signal discrimination through CREB is achieved, explaining why some signals increase CREB phosphorylation without necessarily affecting gene expression.

As above, there are around 4000 CREB target genes in the human genome. Impey *et al* explored the CREB regulon in rat pheochromocytoma cells[442]. Consistent with the diverse functions ascribed to CREB activation, the targets include antioxidants such as MnSOD and UCP-2, anti-inflammatory

or pro-inflammatory genes such as DAF, IL-10 or IL-6, and some with roles in cell survival, such as Bcl-2 and A1[199, 434].

6.1.3 Prior studies of CREB and AMPK, and CREB and HO-1/ MnSOD interaction

AMPK interacts with CREB in two ways. Firstly, it can directly phosphorylate CREB on Ser133, an action which promotes CREB activation[200]. However as explained above phosphorylation may be insufficient for initiation of gene transcription, which also requires binding of transcriptional co-activators. Nevertheless, the interaction has been shown to result in functionally important changes in gene transcription in EC, mediating an increase in DAF expression in response to HUVEC treatment with atorvastatin and rapamycin[206].

Secondly, AMPK can interact with CRTC by phosphorylating them, which would be predicted to cause deactivation and thus reduce CREB-mediated transcription. In *C. elegans*, the AMPK homologue has been shown to phosphorylate the CRTC homologue, thus preventing its translocation to the nucleus and association with CREB. This action promoted longevity[443]. An interaction between AMPK and CRTC has also been shown to inhibit hepatic gluconeogenesis in human cells[444]. Thus AMPK can in different cells and in different circumstances both activate and inhibit CREB-related transcription.

CREB has previously been linked with HO-1 induction in HUVEC. Kronke *et al* showed that HO-1 induction by oxidised phospholipids depends on CREB binding to the CRE site in the HO-1 promoter[203]. Kim *et al* and Chung *et al* have demonstrated that MnSOD induction in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) in a lung cancer cell line requires CREB activation and binding to the MnSOD promoter[445, 446].

6.1.4 Transcriptional regulation of human SOD2

MnSOD is encoded by the *SOD2* gene, which is located on chromosome 6q25 in the human genome (GenBANK accession S77127). The gene is composed of 5 exons and 4 introns, and in addition to the 5' flanking promoter, an enhancer region in the 2nd intron around 2 kbp downstream of the transcription start site has been described (Figure 6.1)[221]. The *SOD2* promoter contains 78% GC nucleotides and does not contain a TATA or CAAT box for initiation of gene transcription.

Constitutive transcription appears to be regulated by the transcription factors specificity protein-1 (SP-1) and activating protein-2 (AP-2), with SP-1 initiating and AP-2 inhibiting transcription[447, 448]. SP-1-dependent promoters are characteristically rich in GC residues, as is the *SOD2* promoter. There are multiple sites for binding SP-1 within the promoter, which is shown in Figure 6.1.

SP-1 is also necessary for inducible expression of MnSOD, but additional transcription factors are also important for induction of MnSOD in response to various stimuli. CREB can bind to a CRE site located around 1200 bp upstream of the transcription start site, and this has been shown in response to

stimulation with TPA[445]. NF κ B can bind both to the distal promoter and also to the intronic enhancer region, and mediates MnSOD induction in response to TNF α and IL-1[224]. FOXO3a, PPAR γ and C/EBP have all also been shown to be involved in MnSOD induction in response to various stimuli across different cell types[221].

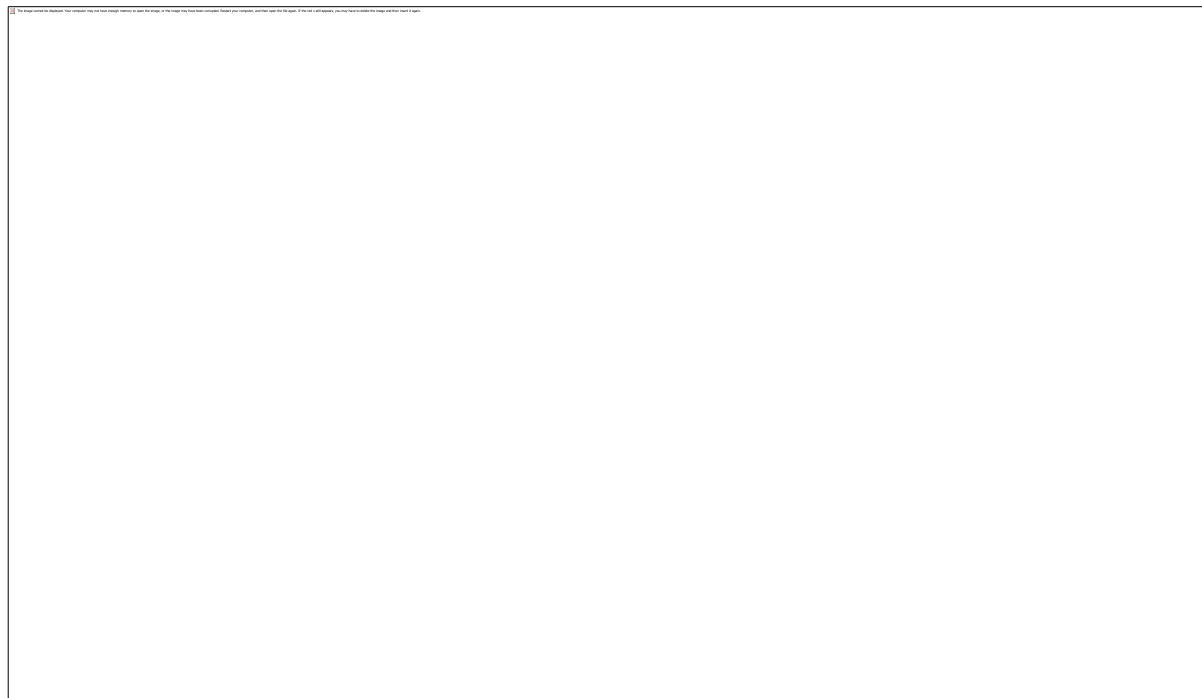


Figure 6.1 Transcription factor binding sites in the SOD2 promoter and intronic enhancer region. Adapted from Miao and St Clair[221].

6.1.5 The role of ROS in MnSOD and HO-1 induction

Normal homeostatic principles of negative feedback would suggest that, given both MnSOD and HO-1 are antioxidant enzymes, elevated levels of ROS might be an important stimulus to their induction, and this has previously been demonstrated in other settings[198, 449]. A further avenue explored in this chapter therefore was the role of ROS in the induction of MnSOD and HO-1 by MTX.

Previous research has shown that MTX increases production of intracellular ROS, at least in macrophage and T cell lines, and does so at concentrations compatible with long term low dose treatment in patients[380]. Several studies have shown that high dose (up to 20 mg/kg) MTX causes oxidative damage *in vivo*[422, 450]. These studies have not consistently been linked however with changes in antioxidant enzyme activity or expression level.

Recent research on ROS biology has shown that the location and extent of ROS production within a cell are important in terms of its significance and effects on cell function, with low levels of superoxide and H₂O₂ production likely being important as a signalling mechanism[136]. MnSOD is located within the mitochondrial matrix where it catalyses the conversion of superoxide to H₂O₂. It is possible therefore that it is specifically mitochondrial ROS production that is most important for its

induction. HO-1 by contrast is predominantly located on microsome membranes within the endoplasmic reticulum, and so might respond more to changes in cytosolic ROS levels.

6.1.6 Chapter Aims

This experiments reported in this chapter sought first to establish the role of AMPK activation in the induction of MnSOD and HO-1 by MTX, using predominantly a loss-of-function approach. Secondly, the role of CREB in mediating transcription in response to AMPK activation was investigated in 3 ways: phosphorylation in response to MTX; effect of CREB depletion by RNA interference on MnSOD and HO-1 induction; and ChIP to explore CREB binding to the *SOD2* promoter. Finally, the role of ROS signalling was explored by establishing firstly whether MTX increases intracellular ROS levels, and secondly whether abolishing ROS production affects HO-1 and MnSOD expression in response to MTX.

6.2 Results

6.2.1 Inhibition of AMPK reduces MTX-induced HO-1 and MnSOD mRNA expression but not protein

In order to explore whether AMPK phosphorylation is necessary for the induction of MnSOD and HO-1 by MTX, Compound C, a pharmacological inhibitor of AMPK activation, was used. HUVEC were treated with 5 μ M Compound C 30 minutes before the addition of MTX 100nM for 48h. The addition of Compound C significantly abrogated the MnSOD and HO-1 mRNA response to MTX (Figure 6.2A and B). However, no effect was seen on MnSOD and HO-1 protein levels, and moreover Compound C alone caused an induction of MnSOD and HO-1 protein (Figure 6.2C-F). This has since been reported in the literature in relation to HO-1 expression[451]. Given that the study of HO-1 induction by Compound C reported that this occurred independently of its effects on AMPK activity, it seemed likely that the explanation for the results of these experiments was that Compound C caused an acute induction of MnSOD and HO-1 mRNA, which then resolved. However, its inhibition of AMPK signalling remained, thus preventing the later induction of MnSOD and HO-1 mRNA by MTX. However, the induction of MnSOD and HO-1 protein by Compound C, being longer-lasting, obscured the response to MTX. Therefore, AMPK depletion by RNA interference was carried out to investigate its role in MTX induction of cytoprotective genes more specifically.

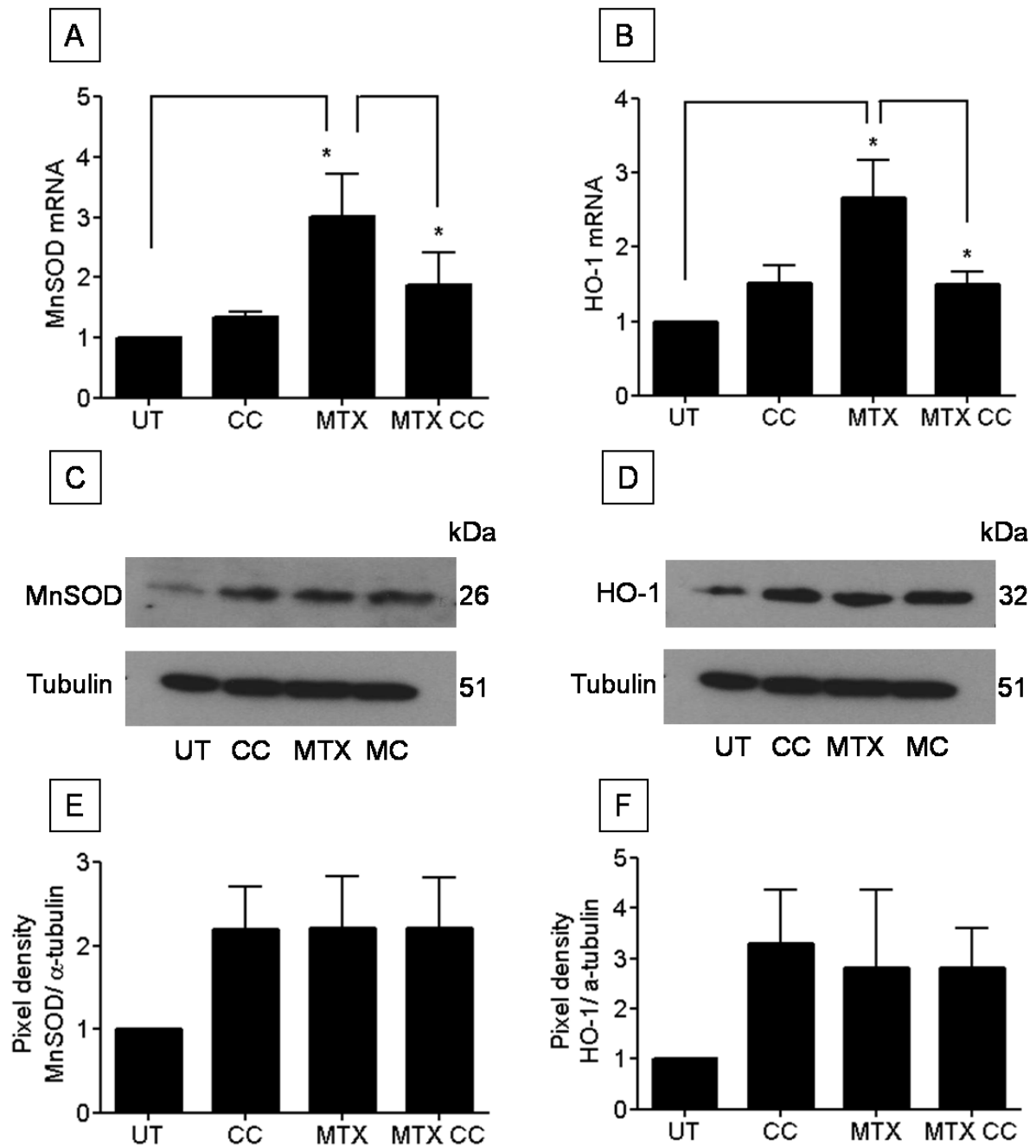


Figure 6.2 Pharmacological AMPK inhibition reduces induction by MTX of MnSOD and HO-1 mRNA, but not protein. HUVEC were treated with MTX 100nM and the AMPK inhibitor Compound C 5 μ M for 48h. A and B: change in MnSOD and HO-1 mRNA. C and D: representative western blots. E and F: densitometry of western blots. Data shown represent the results of at least 3 experiments. UT: vehicle control CC: compound C; MTX CC and MC: MTX and Compound C together. * = $p < 0.05$.

6.2.2 Depletion of AMPK α_1 or AMPK α_2 inhibits induction of HO-1 and MnSOD by MTX

Given that pharmacological inhibition of AMPK signalling was not successful in demonstrating whether AMPK activation is required for MnSOD induction by MTX, an alternative loss of function approach was adopted. AMPK signalling was inhibited using RNA interference. HUVEC were transfected with siRNA directed against the AMPK α_1 or α_2 subunit, which contain the phosphorylation site for activation, and then treated with MTX for 48h. The efficiency of knockdown was difficult to assess by western blot as the anti-AMPK α antibody used did not distinguish between the α_1 and α_2 isoforms. A reasonable degree of knock-down was however achieved when measured by qRTPCR (Figure 6.3C and D).

Induction of MnSOD mRNA and protein by MTX was significantly attenuated by AMPK α_2 siRNA and by α_1 and α_2 siRNA together (Figure 6.3A and 6.4A and B). Similar analysis of HO-1 expression showed that depletion of either the α_1 or α_2 subunit of AMPK inhibited mRNA and protein induction (Figure 6.3B and 6.4A and C), but depletion of both together resulted paradoxically in an increase in HO-1 mRNA expression levels, although not protein.

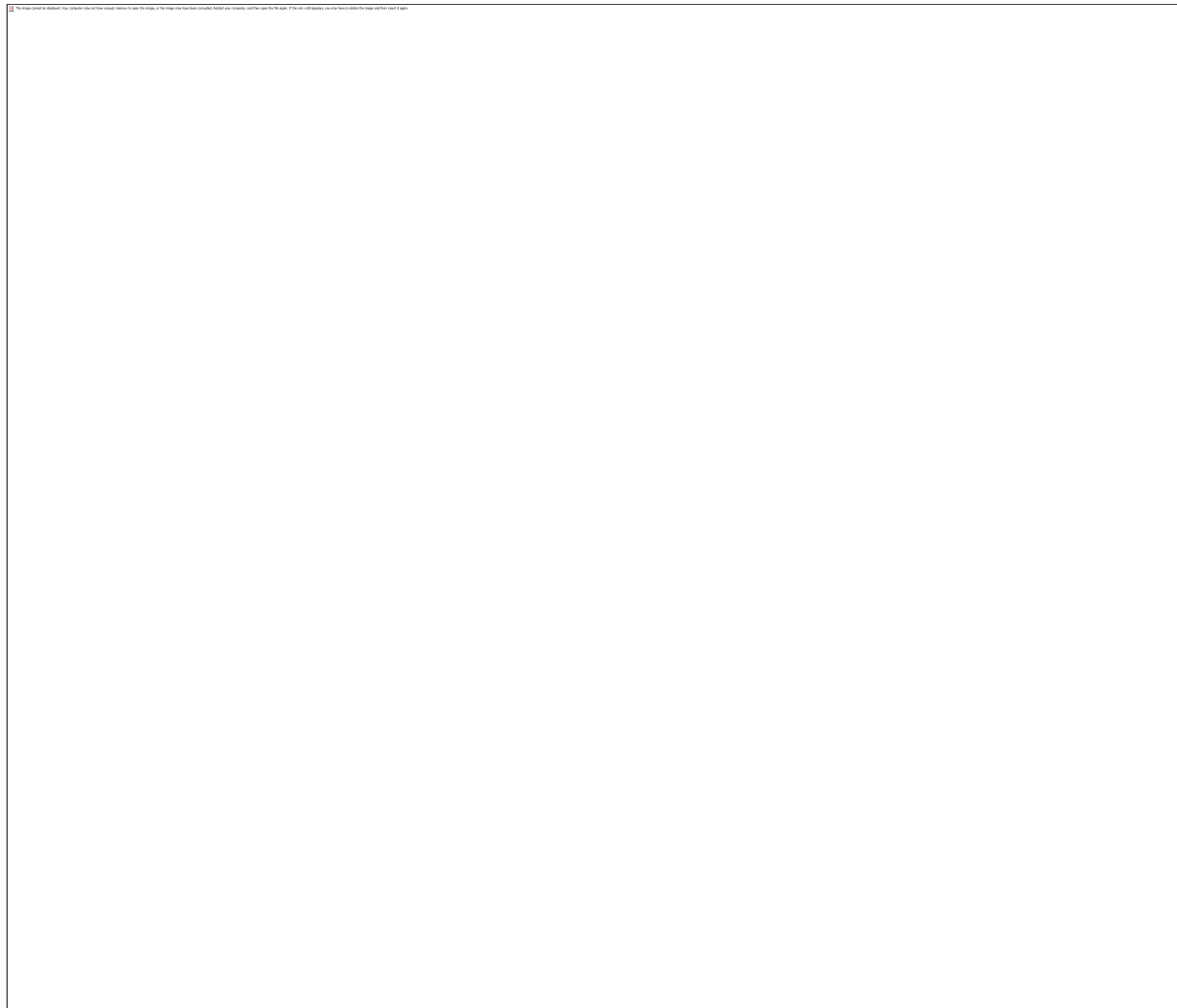


Figure 6.3 RNA interference with siRNA directed against the $\alpha 1$ and $\alpha 2$ subunits of AMPK prevents the induction of MnSOD and HO-1 mRNA by MTX. HUVEC were transfected with 40nM siRNA directed against the $\alpha 1$ and $\alpha 2$ subunits of AMPK or scramble control sequences, then treated with MTX 100nM for 48h. A: MnSOD mRNA levels relative to those of cells treated with control siRNA; B: HO-1 mRNA levels; C: AMPK $\alpha 1$ mRNA levels; D: AMPK $\alpha 2$ mRNA levels. C: control siRNA; CM: control siRNA and MTX 100nM; $\alpha 1$: AMPK $\alpha 1$ siRNA; $\alpha 1$ M: AMPK $\alpha 1$ siRNA and MTX 100nM; $\alpha 2$: AMPK $\alpha 2$ siRNA; $\alpha 2$ M: AMPK $\alpha 2$ siRNA and MTX 100nM; $\alpha 12$: AMPK $\alpha 1$ and AMPK $\alpha 2$ siRNA; $\alpha 12$ M: AMPK $\alpha 1$ and AMPK $\alpha 2$ siRNA and MTX 100nM. Data shown are the results of 3 experiments. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

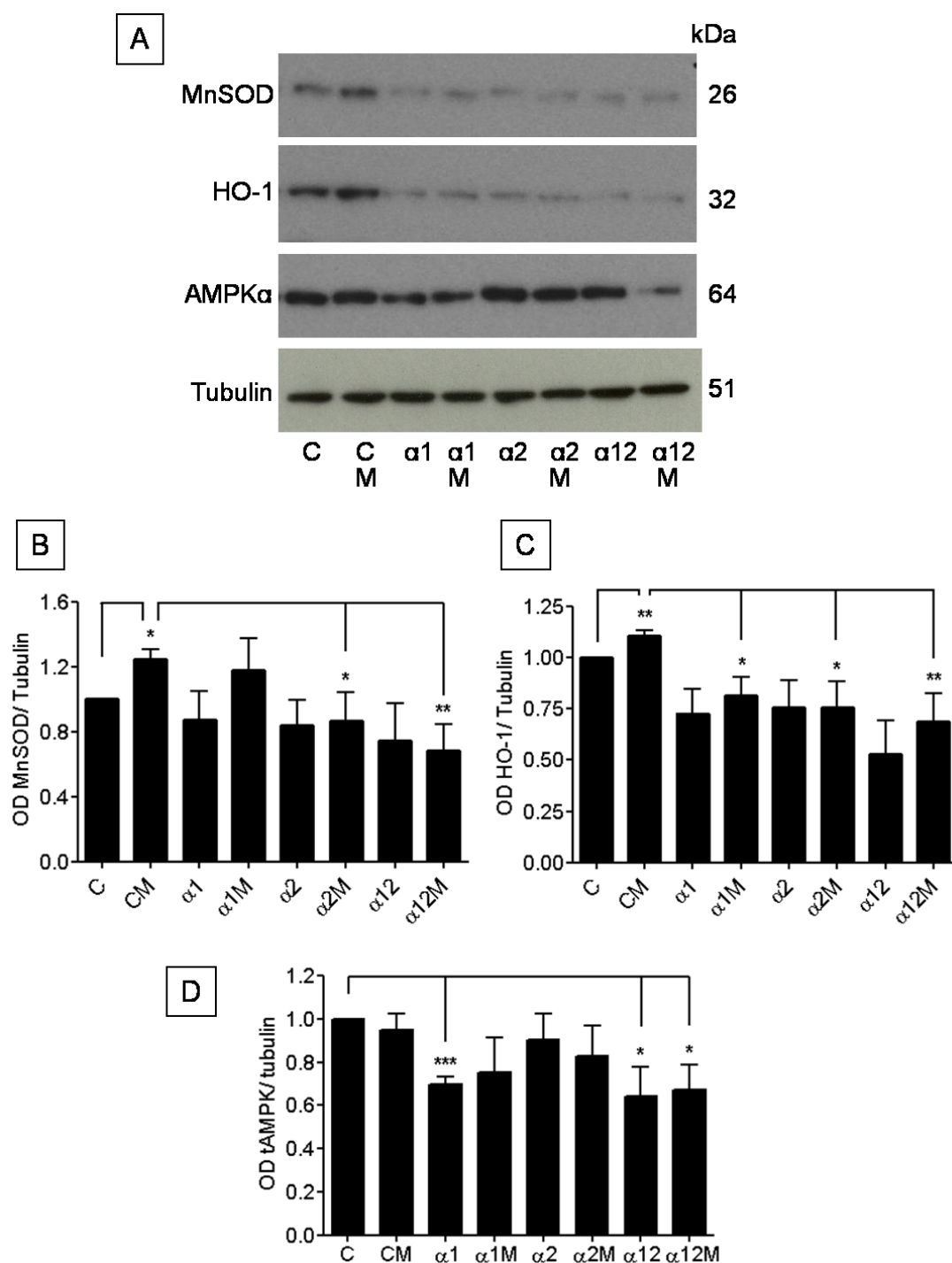


Figure 6.4 Inhibition of AMPK signalling with siRNA targetting the $\alpha 1$ and $\alpha 2$ subunits reduces induction of MnSOD and HO-1 protein by MTX. HUVEC were transfected with control, AMPK $\alpha 1$, AMPK $\alpha 2$ or AMPK $\alpha 1$ and $\alpha 2$ siRNA together then treated with MTX 100nM for 48h. A: representative Western blot; B: densitometry of MnSOD expression; C: densitometry of HO-1 expression; D: densitometry of AMPK α expression to show knockdown efficiency. C: control siRNA; CM: control siRNA and MTX 100nM; $\alpha 1$: $\alpha 1$ siRNA; $\alpha 1M$: $\alpha 1$ siRNA and MTX 100nM; $\alpha 2$: $\alpha 2$ siRNA; $\alpha 2M$: $\alpha 2$ siRNA and MTX 100nM; $\alpha 12$: $\alpha 1$ and $\alpha 2$ siRNA ; $\alpha 12M$: $\alpha 1$ and $\alpha 2$ siRNA and MTX 100nM. Data shown are the results of 6 experiments. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

6.2.3 AMPK activation with AICAR induces MnSOD expression

To confirm the role of AMPK in expression of cytoprotective genes, HUVEC were treated with the AMPK activator AICAR. AICAR is converted to the purine nucleotide ZMP intracellularly, which is capable of binding to the regulatory γ subunit and allosterically activating AMPK in the same fashion as AMP. An induction of MnSOD protein was observed with 1mM AICAR after 16-24h (Figure 6.5), as has previously been published[262], lending further support to the hypothesis that MTX activation of MnSOD is dependent on AMPK activation.

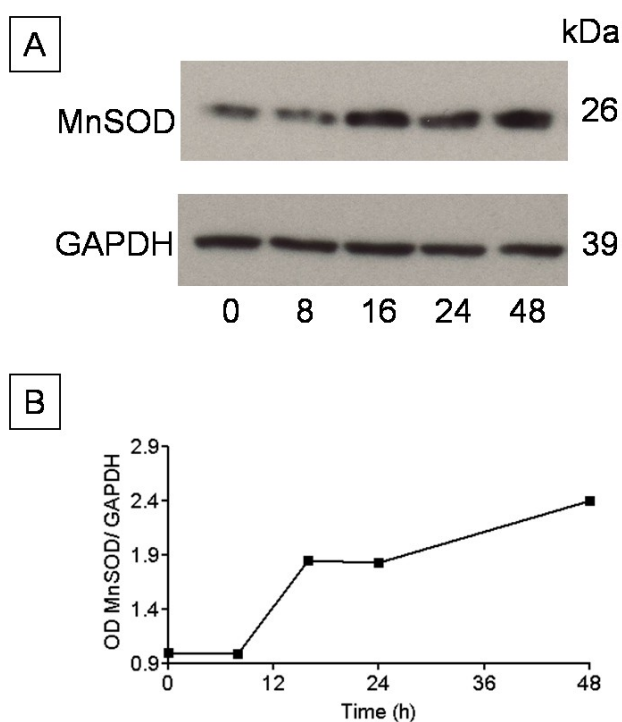


Figure 6.5 AMPK activation using AICAR leads to induction of MnSOD protein. HUVEC were treated with AICAR 1mM over the course of 48h. A: western blot image of MnSOD induction. B: Densitometry results. Data shown is the result of a single experiment. OD: optical density.

6.2.4 MTX treatment causes phosphorylation of CREB in EC

The transcription factor CREB is known to be a direct target of AMPK activation[200], and has also been shown to bind to the *SOD2* promoter and activate transcription[276, 445]. Moreover, its activity is positively associated with vascular health[201]. It was therefore considered a good candidate to study as a potential intermediary in the induction of MnSOD by AMPK activation with MTX stimulation. CREB is activated by phosphorylation at Ser133. By western blot, a significant induction of phosphorylated CREB^{Ser133} was seen when HUVEC were treated for 48h with MTX 10nM and 100nM (Figure 6.6).

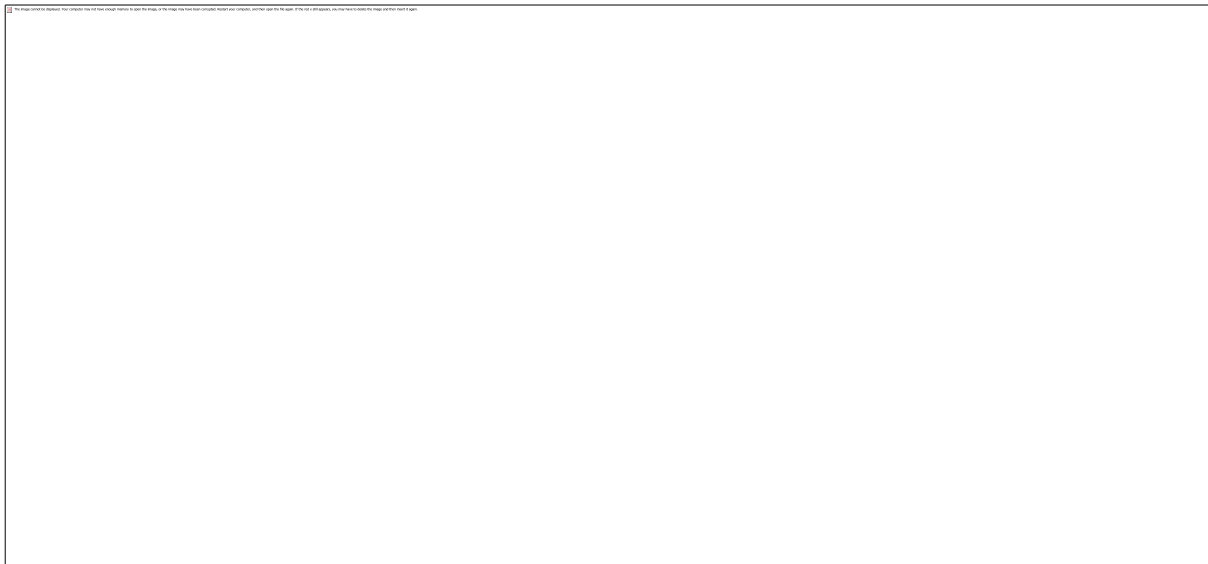


Figure 6.6 MTX causes CREB phosphorylation. HUVEC were treated with MTX or DMSO vehicle control at increasing concentrations for 48h. A: representative western blot; B: corresponding densitometry. Data shown are the results of >3 experiments. UT = vehicle control; *=p<0.05.

6.2.5 Disruption of AMPK with siRNA inhibits CREB phosphorylation by MTX

In order to establish whether CREB was activated by AMPK in HUVEC treated with MTX, CREB phosphorylation was examined in HUVEC treated with AMPK α 1 and α 2 siRNA. CREB^{Ser133} phosphorylation was abrogated by AMPK α 1 or α 2 siRNA, suggesting a linear signalling pathway (Figure 6.7). This is consistent with the published literature suggesting that CREB is a direct target of AMPK activation[200].

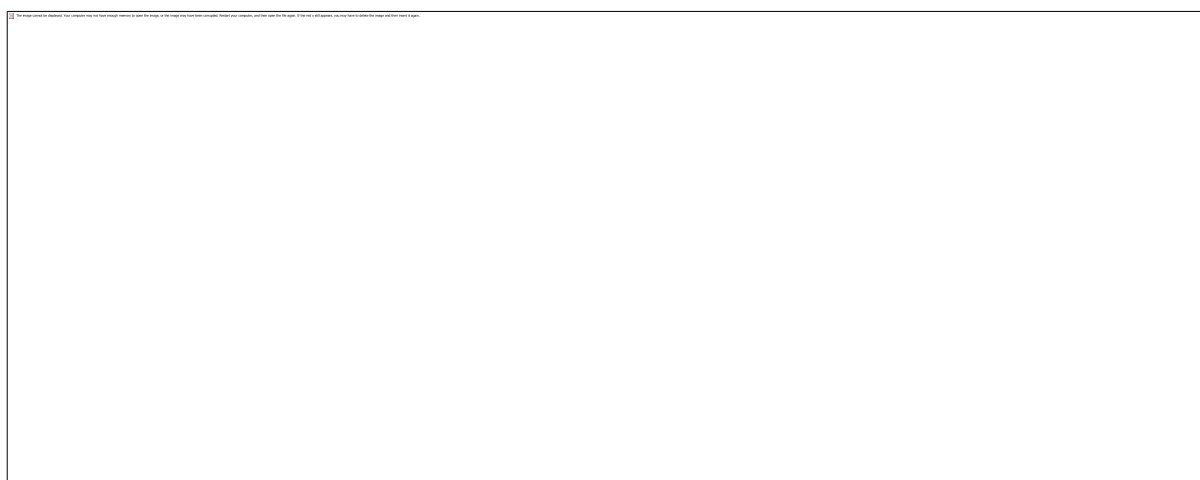


Figure 6.7 Disruption of AMPK signalling with siRNA targetting the α 1 and α 2 subunits inhibits CREB phosphorylation by MTX. HUVEC were transfected with control, AMPK α 1, AMPK α 2 or AMPK α 1 and α 2 siRNA together then treated with MTX 100nM for 48h. A: representative western blot; B: densitometry of CREB expression. Knockdown efficiency is shown in Figure 5.2. C: control siRNA; CM: control siRNA and MTX 100nM; α 1: α 1 siRNA; α 1M: α 1 siRNA and MTX 100nM; α 2: α 2 siRNA; α 2M: α 2 siRNA and MTX 100nM; α 12: α 1 and α 2 siRNA ; α 12M: α 1 and α 2 siRNA and MTX 100nM. Data shown are the results of 6 experiments. * = $p < 0.05$; ** = $p < 0.01$.

6.2.6 CREB depletion reduces MnSOD and HO-1 induction by MTX

Next, in order to explore whether CREB is required for the induction of MnSOD and HO-1 by MTX, CREB was inhibited using RNA interference. A pool of 5 siRNA sequences directed against CREB was transfected into HUVEC for 16h before treatment with MTX 100nM for 48h. This resulted in a reduction of CREB protein by over 80% (Figure 6.8C). MnSOD and HO-1 mRNA levels were significantly reduced in HUVEC treated with CREB siRNA and MTX, compared with those treated with MTX after transfection with scramble control siRNA (Figure 6.8A and B). This suggests that CREB protein is required for the induction by MTX of MnSOD and HO-1, and that this forms an important intermediary in AMPK- mediated cytoprotection.

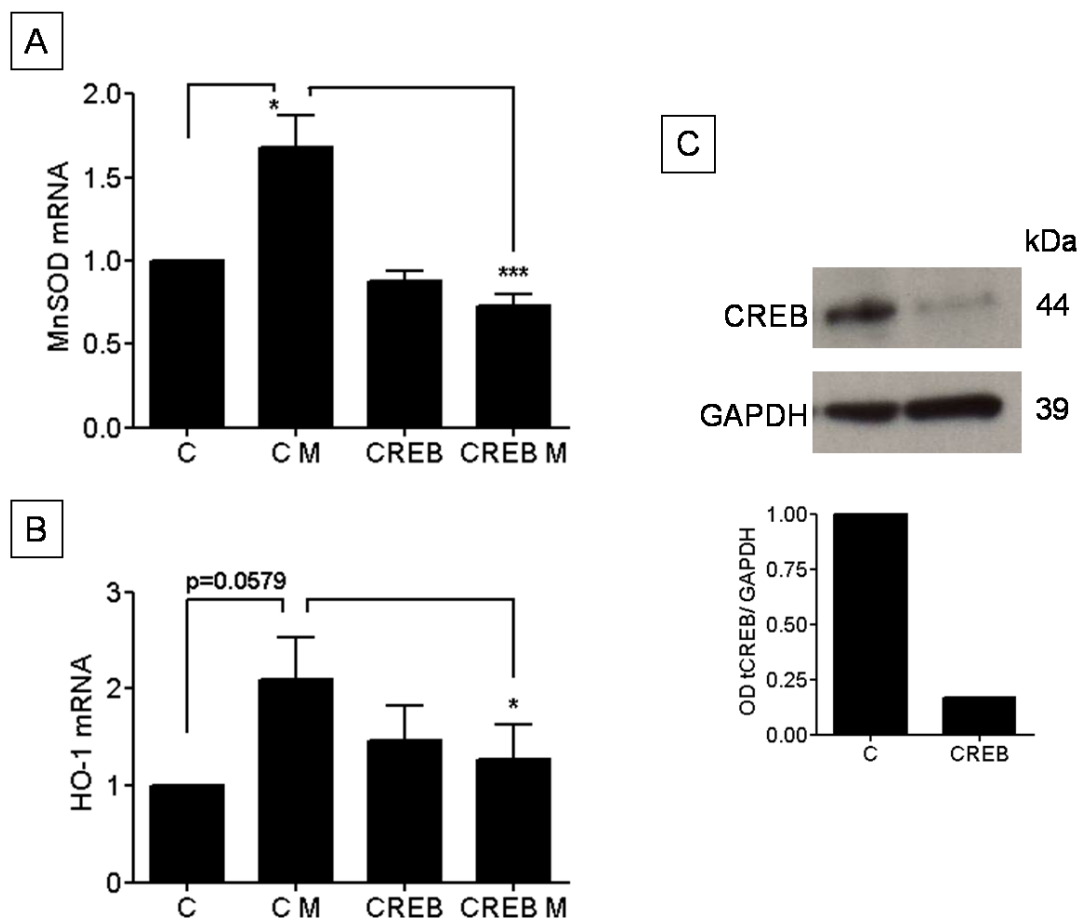


Figure 6.8 Inhibition of CREB prevents induction of MnSOD and HO-1 mRNA by MTX. CREB was inhibited with pooled CREB siRNA, before stimulation with MTX 100nM for 48h. A and B: results of qRT-PCR for MnSOD and HO-1 mRNA. C: Western blot demonstrating efficiency of CREB knockdown. C: control siRNA; M: MTX 100nM; CREB: CREB siRNA; tCREB: anti-CREB antibody. A and B represent the results of 6 experiments; C shows the results of 1 experiment. *= $p < 0.05$; ***= $p < 0.001$.

6.2.7 MTX treatment causes CREB binding to the *SOD2* promoter

The final step was to look for enhanced binding of CREB to the *SOD2* promoter in HUVEC treated with MTX. This was done using chromatin immunoprecipitation. Analysis of the *SOD2* promoter (GenBank accession number AF059197) using MatInspector software[392] identified 2 strong potential CREB binding sites (Figure 6.9). Direct demonstration of CREB binding to the *SOD2* promoter has previously been shown for the more proximal half site[445]. A significant increase in the binding of CREB to the MnSOD promoter following MTX treatment was found using primers designed to capture this site (Figure 6.10), although a trend towards enhanced binding was also seen with primers designed around the more distal site. This is understandable, as these sites are within 300 kbp of each other and therefore may have been pulled down with each other given that average chromatin shearing was 500bp segments. No significant enhanced binding was detected with primers designed at a downstream site 2kbp further away (data not shown).

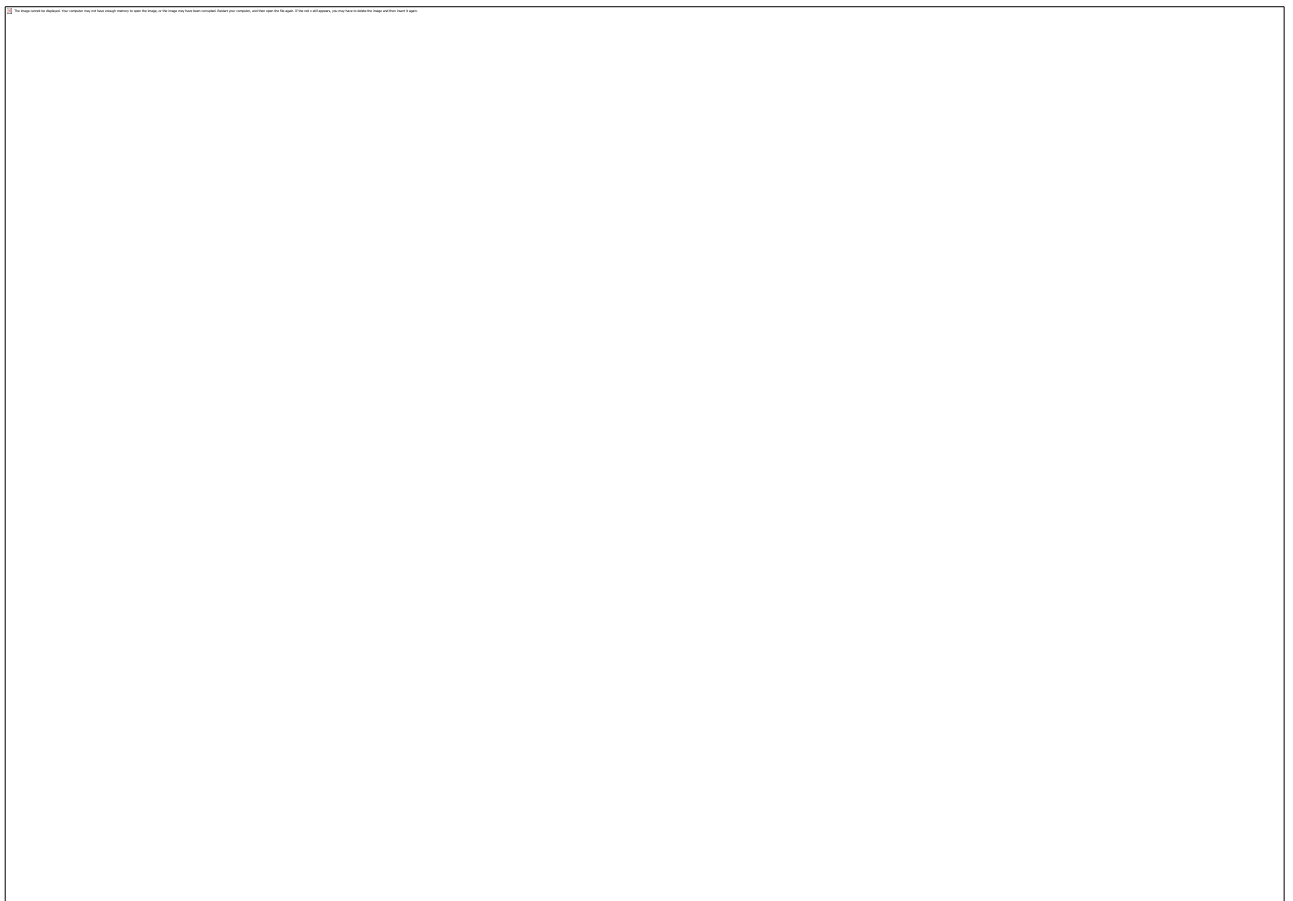


Figure 6.9 *SOD2* promoter sequence. Putative CREB binding sites are marked in red, and the transcription start site in blue.

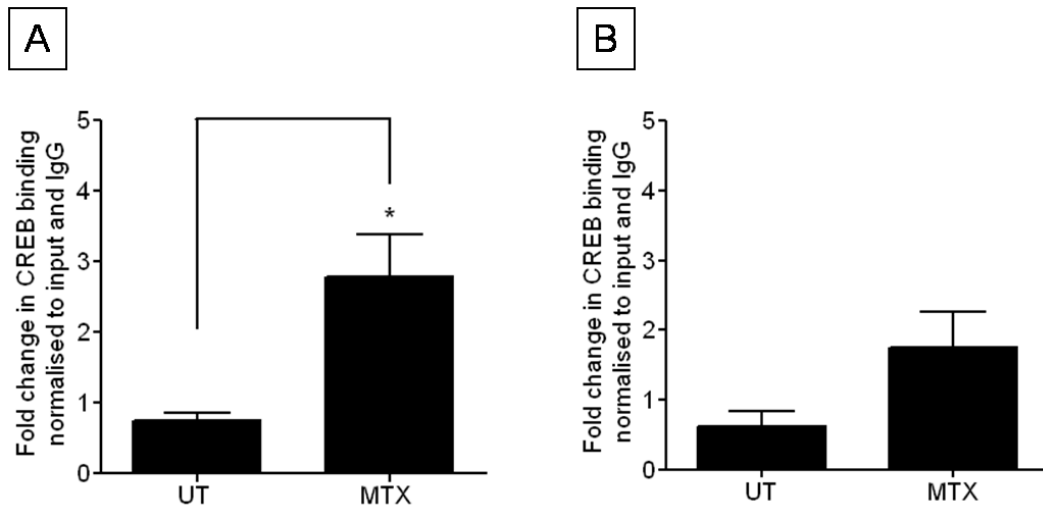


Figure 6.10 MTX causes an increase in CREB-1 binding to the *SOD2* promoter. Pooled HUVEC were stimulated with MTX 100nM for 48h before chromatin immunoprecipitation for CREB-1. Data shown are the fold change in transcript level detected by qPCR, normalised to input DNA and IgG control for each condition. Data represent the results of 5 experiments. UT = vehicle control; * = $p < 0.05$.

6.2.8 Role of ROS in MnSOD and HO-1 induction by MTX

There is evidence suggesting that MTX treatment promotes ROS production and that high dose MTX causes oxidant damage to tissues. Indeed, production of ROS has been proposed as a major mechanism of action to explain MTX's anti-inflammatory efficacy[380]. Conversely, other work suggests that MTX reduces ROS with one study demonstrating a reduction in IL-6-induced ROS in synovial tissue treated with MTX[381]. I therefore sought to explore whether MTX alters ROS production in HUVEC, whether its effects are predominantly on mitochondrial ROS generation or other sources, and whether ROS production is an important intermediate step in AMPK activation or induction of anti-oxidant genes by MTX.

First, I looked for evidence of ROS production in HUVEC treated with MTX 100nM for 48h. HUVEC were loaded with the ROS reporter dye CM-H2DCFDA and fluorescence measured by flow cytometry. A 50% increase in ROS generation was observed in HUVEC treated with MTX, although this was highly variable. This compares with a doubling in ROS levels in cells treated with 10 μ M H₂O₂ for 1h (Figure 6.11A and B).

The next step was to study whether ROS production was important for the induction of the anti-oxidant genes HO-1 and MnSOD. HUVEC were pre-treated with the ROS scavenger NAC for 1h prior to MTX 100nM for 48h. NAC is a derivative of cysteine with an acetyl group added to the nitrogen atom. It is a precursor to glutathione, thus treating cells with NAC increases the pool of glutathione available. The use of NAC inhibited HO-1 mRNA induction but not MnSOD (Figure 6.11C and E).

Finally, I explored the specific role of mitochondrial ROS, by pre-treating HUVEC with the specific mitochondrial ROS scavenger mitoTEMPO for 30 minutes before the addition of MTX. This did not alter MnSOD or HO-1 mRNA induction (Figure 6.11D and F). However, this may be a technical failure rather than a true result: the experiment lacked a suitable gene response to demonstrate an effect of mitochondrial ROS scavenging.



Figure 6.11 MTX increases ROS production, but abolishing ROS affects induction of HO-1 mRNA only and not MnSOD. A and B: HUVEC stimulated with MTX or H_2O_2 were loaded with CM-H2DCFDA and fluorescence measured by FACS. Yellow: unstained HUVEC; pale blue: unstained HUVEC treated with MTX; dark blue: untreated HUVEC loaded with CM-H2DCFDA; red: HUVEC treated with H_2O_2 10 μ M and DCF-DA; green: HUVEC treated with MTX 100nM and CM-H2DCFDA. C and D: HO-1 mRNA was measured after pre-treatment with NAC 10mM or Mitotempo 10 μ M and MTX 100nM. E and F: MnSOD mRNA was measured after pre-treatment with NAC 10mM or Mitotempo 10 μ M and MTX 100nM. UT = vehicle control; M: MTX 100nM; mito: mitoTEMPO 10 μ M; * = $p < 0.05$; *** = $p > 0.001$.

6.3 Discussion

In this chapter I have presented data exploring the mechanisms by which MTX induces cytoprotective gene expression in EC. The induction of MnSOD and HO-1 is dependent on AMPK activation; shown by inhibition of AMPK both pharmacologically and using RNA interference. The involvement of the transcription factor CREB has also been established: MTX treatment results in CREB phosphorylation, which is abolished when AMPK is inhibited, thus demonstrating that CREB activation lies downstream of AMPK. Inhibition of CREB using siRNA prevented the MTX-mediated induction of MnSOD and HO-1, and ChIP was used to show direct binding of CREB to the MnSOD promoter. Finally, the role of ROS was studied. MTX treatment of HUVEC resulted in a modest increase in intracellular ROS, detected using the reporter dye CM-H₂DCFDA. Inhibition of generalised intracellular ROS production using NAC prevented the induction of HO-1 but not MnSOD mRNA. However, neither was altered by the addition of the specific mitochondrial ROS scavenger mitoTEMPO.

6.3.1 AMPK is required for MTX-mediated induction of MnSOD and HO-1

The initial experiments presented in this chapter demonstrated that AMPK is required in order for MTX treatment of HUVEC to induce MnSOD and HO-1 expression. Two loss-of-function techniques were used: firstly AMPK was inhibited pharmacologically using Compound C, and secondly RNA interference was performed to deplete selectively AMPK α 1, AMPK α 2, or both together.

Pharmacological inhibition with Compound C did reduce the MTX-mediated induction of MnSOD and HO-1 mRNA, but not of protein. Indeed, treatment with Compound C alone increased expression of HO-1 and MnSOD protein. This has subsequently been reported for HO-1, as an effect independent of its AMPK-inhibiting properties[451]. It is therefore possible that Compound C stimulated an induction of HO-1 and MnSOD. mRNA transcription perhaps peaked and fell while inhibition of AMPK remained, thus allowing the delayed, MTX-mediated induction of MnSOD and HO-1 mRNA to be seen. However, protein expression induced by Compound C remained present at 48h, thus obscuring any effects of MTX mediated via AMPK.

Inhibition of AMPK signalling using RNA interference carried several advantages over pharmacological inhibition. Firstly, the effects of depleting either the α 1 or α 2 isoform, or both together, could be explored. Secondly, the targeting of one specific mRNA transcript means that this technique is much more specific.

siRNA depletion of AMPK α 1, AMPK α 2 or both together prevented the MTX-mediated induction of MnSOD and HO-1 mRNA and protein, with a couple of caveats. Firstly, the effects on MnSOD reached statistical significance when the AMPK α 2 isoform or both together were depleted, although

there was only a trend towards reduction in MnSOD induction with siRNA directed against AMPK α 1. This may be due to technical limitations; it is possible that the other isoform compensates for the loss of one when either is depleted. Alternatively, it may reflect the mechanism by which MTX is likely to activate AMPK. Salt *et al* have shown that AMPK α 2 is more sensitive to AMP than AMPK α 1[264], thus speculatively it may be that AMPK α 2 is more responsive to MTX's effects on AMP and ZMP levels.

Previous studies have shown that the AMPK α 1 isoform is more abundant in EC and thus have focused on its functional effects[262, 276]. More recently, however, evidence has emerged suggesting that important vasculoprotective effects are also mediated through AMPK α 2[331]. Our study suggested that the effects of MTX on MnSOD induction were predominantly mediated through AMPK α 2, although depletion of either α subunit attenuated the responses.

Secondly, although HO-1 induction by MTX was significantly inhibited when either AMPK α 1 or AMPK α 2 was depleted, when both were depleted there was a paradoxical rise in HO-1 mRNA and protein, and also in CREB phosphorylation. I think this is likely to represent a stress response following a profound loss of AMPK α : while the other isoform may be able to compensate partially for the loss of either α 1 or α 2, loss of both may alter other intracellular signalling more profoundly and may have consequences for cellular metabolism that loss of a single isoform does not. It suggests that AMPK is not necessary and sufficient for HO-1 induction or for CREB phosphorylation, even if it is capable of it, but MnSOD induction is not possible without AMPK functioning. This is not surprising given the large number of other mechanisms of HO-1 induction and of CREB phosphorylation previously identified. However, it is difficult to draw firm conclusions given that the siRNA experiments were performed with a single sequence and so could represent an off-target effect.

Several other technical limitations to these experiments should be noted. Firstly RNA interference was performed using only a single siRNA sequence for each isoform. It is therefore theoretically possible that the effects observed relate to an off-target effect of that sequence. However, the finding that depletion of either isoform gave broadly the same result, and that pharmacological inhibition of AMPK produced similar findings in mRNA results, suggests that these results are valid. Secondly, the quantification of the AMPK α isoform knockdown by western blot is not very impressive. This is probably because the antibody used was not specific for α 1 or α 2 isoform. Finally, the degree of MnSOD and HO-1 induction seen with MTX treatment in cells transfected with scrambled control siRNA was much reduced compared to that seen in untransfected cells. This may reflect the process of transfection itself, which is presumably stressful to cells, and so led to an induction in MnSOD and HO-1 (data not shown), and thus there was limited scope to show an additional induction beyond this.

In view of the limitations described above, a further approach was taken to establish the role of AMPK in induction of MnSOD. HUVEC were treated with the AMPK activator AICAR and this led

to a two fold induction in MnSOD protein expression, thus providing additional supportive evidence that AMPK activation leads to MnSOD expression. This data confirms previously reported findings[262, 310]. Therefore, it seems reasonable to conclude that MTX activation of AMPK leads to the induction of the cytoprotective genes MnSOD and HO-1.

6.3.2 CREB is activated by MTX via AMPK and promotes MnSOD expression

The second set of experiments reported in this chapter explored the role of the transcription factor CREB in the MTX-AMPK-mediated induction of MnSOD and HO-1. A variety of techniques was employed to do this. Firstly, it was established that CREB^{Ser133} phosphorylation is increased in HUVEC treated with MTX. Secondly, it was shown that AMPK α 1 or α 2 depletion prevented this, suggesting that CREB lies downstream of AMPK. Thirdly, depletion of CREB by RNA interference completely abrogated the induction of MnSOD and HO-1 mRNA by MTX. Lastly, ChIP demonstrated enrichment of CREB on the *SOD2* promoter in HUVEC treated with MTX.

CREB^{Ser133} phosphorylation was first studied in HUVEC treated with increasing concentrations of MTX. Although a statistically significant increase in phosphorylation was seen at 10nM and 100nM MTX, there was no dose-response relationship, and in fact even very low concentrations of MTX (0.1nM) may have had some effect. This makes the observation more likely to be relevant to patients, if even very low concentrations of MTX exert biological effects observable *in vitro*.

It was also demonstrated that CREB^{Ser133} phosphorylation is abrogated by depletion of AMPK α 1 or α 2 siRNA. This was expected, given previous reports that CREB is a direct phosphorylation target of AMPK[200]. Similar to the effects on HO-1, however, depletion of both AMPK α isoforms together resulted in a paradoxical increase in phosphorylation. This may also represent a stress response; certainly a number of other kinases are also capable of CREB phosphorylation, particularly PKA[434].

Finally, the role of CREB in the MTX-mediated induction of MnSOD and HO-1 was explored. Depletion of CREB by RNA interference abolished the induction of MnSOD and HO-1 mRNA, and ChIP demonstrated increased binding of CREB to the *SOD2* promoter in MTX-treated cells. Due to time limitations, ChIP was not performed to look for binding of CREB to the HO-1 promoter. A previous study of MnSOD induction in cancer cells by TPA demonstrated that CREB binding to the MnSOD promoter enhanced MnSOD transcription[445]. A reporter assay employing a series of reductions in promoter size established that the responsible site was the CRE half site identified in my experiments by MATInspector, and it was with primers designed to pick up this site that I detected an increase in CREB binding to the MnSOD promoter in the ChIP assay.

However, neither CREB occupancy of the MnSOD promoter nor increased CREB phosphorylation with MTX treatment means that activation of transcription by CREB in response to MTX has been

established conclusively. Firstly CREB phosphorylation has previously been shown not to be sufficient to drive transcription; recruitment of co-activators is also required[433]. Secondly, a large number of genes have CREB occupying their promoters without it necessarily promoting gene expression[434]. Ideally, a luciferase reporter assay should also have been performed to investigate directly whether MTX treatment leads to enhancement of MnSOD transcription via CREB, and also perhaps co-immunoprecipitation to look for enhanced binding of CREB to its coactivators in the presence of MTX. Nevertheless, the complete abolition of MnSOD or HO-1 induction with CREB depletion does suggest its presence is necessary for this to occur.

Although I only studied the role of CREB in the induction of MnSOD and HO-1 expression, it is also likely to be important for the expression of a number of the other genes induced by MTX in Chapter 5. UCP-2 and Bcl-2 also contain CRE sites in their promoters, while Hamdulay *et al* showed using a luciferase reporter assay that CREB promotes DAF transcription in response to atorvastatin and rapamycin[206, 434]. A1 induction via CREB has been studied in the context of microbe-induced macrophage apoptosis. Park *et al* did not observe an interaction, although Hsu *et al* did following activation of the same precursor signalling pathways[452, 453].

I have demonstrated that AMPK inhibition prevents CREB phosphorylation, and thus conclude that this indicates a pathway whereby MTX activates AMPK, which in turn activates CREB and alters gene expression. However, CREB activation may also occur via adenosine. Adenosine exerts anti-inflammatory effects by binding to A_{2A} receptors, G-protein-coupled receptors which signal via cAMP[372]. Given that CREB activation in most circumstances occurs through changes in cAMP levels resulting in PKA activation, this is definitely possible. However, the genes activated by CREB in response to changes in cAMP all contain TATA boxes in their promoters, and CRE sites within 250bp of that TATA box[436, 437]. This is not the case with *SOD2*, where the promoter CRE sites are 1200 - 1500bp distal to the transcription start site, and there is no TATA box[221]. Therefore, *SOD2* would seem not to have the characteristics of a gene capable of responding via CREB to changes in cAMP, suggesting that changes in adenosine might not be so important for its induction by MTX.

Given that CREB-mediated activation of gene transcription is thought only to occur via cAMP in genes with TATA boxes, and then only when bound to CRE sites within 250bp of a TATA box, a question remains as to how CREB activates transcription in genes where it binds to more distal promoter regions, and in genes with no TATA box such as *SOD2*. It is known that it is still capable of activating transcription in these circumstances, as was revealed by MnSOD induction in response to TPA[445]. The *SOD2* gene forms loops, bringing together the promoter and the intronic enhancer to allow NFκB binding to the intronic enhancer to activate transcription[454]. Speculatively, a similar loop could also form between proximal and distal regions of the promoter itself to allow distal CREB binding to activate transcription.

6.3.3 The contribution of ROS to MnSOD and HO-1 induction

The final section of experiments presented in this chapter concerned the role of ROS in the induction by MTX of MnSOD and HO-1. Firstly, it was established that treating HUVEC with low concentrations of MTX caused a small rise in intracellular ROS levels, detected by the non-specific ROS reporter dye CM-H₂DCFDA. Secondly, treating HUVEC with NAC to abolish ROS production completely prevented HO-1 induction by MTX, but did not alter MnSOD induction. Finally, treating HUVEC with mitoTEMPO, a mitochondrial-specific ROS scavenger, did not alter either MnSOD or HO-1 induction.

It was already known that treating macrophages and T-cells with doses of MTX similar to those used in these experiments results in increased ROS production and promotes apoptosis of activated cells[380]. I showed that, although a modest increase in ROS does occur in HUVEC treated with MTX, as demonstrated in Chapter 5, MTX treatment of relatively quiescent cells in culture does not increase apoptosis. MTX may do this through an effect on glutathione metabolism: high doses of MTX, at least, inhibit glutathione reductase and may thus reduce the intracellular pool of reduced glutathione[416]. It remains unclear whether the increase in ROS is an unwanted effect of MTX or whether it is a useful signalling phenomenon that is essential to the changes in gene expression and cell metabolism promoted by it.

Treating HUVEC with a high concentration of NAC should have prevented MTX exerting any effect on the pool of reduced glutathione available for redox reactions, and so the abolition of HO-1 induction by the addition of NAC was unsurprising, given that HO-1 is a gene highly responsive to changes in ROS. It contains ARE sites in its promoter and its transcription is closely regulated by the ROS-responsive transcription factor Nrf-2[140, 430].

What was surprising to me was that MnSOD induction was not altered; indeed NAC alone led to a small induction. This may reflect the subcellular localisation of MnSOD in the mitochondrial matrix: it is therefore not involved in the regulation of redox processes in the cytosol and thus its expression may not change in response to changes in the cytosolic concentration of reduced glutathione. Instead MnSOD is likely to be important for regulating ROS levels within the mitochondria, and given that EC do not generally rely on mitochondria for energy production, changes in endothelial mitochondrial ROS are likely to have a signalling function rather than just be a by-product of necessary respiration[120, 455]. To explore this HUVEC were treated with the mitochondrial-specific ROS scavenger mitoTEMPO, but this also did not change the induction of MnSOD by MTX. Although this may exclude a role for ROS in MnSOD induction, a technical failure due to too low a concentration of mitoTEMPO cannot be excluded in the absence of a positive control. Of note, my colleague Fahad Alrashid has since shown that mitoTEMPO 20 μ M inhibits celecoxib-mediated HO-1 induction (personal communication).

7 MTX TREATMENT OF WBF1 MURINE VASCULOPATHY

7.1 Introduction

The work presented in this thesis thus far has demonstrated that laminar shear stress and VEGF, known endogenous endothelial protective factors, can activate AMPK signalling in HUVEC, as can MTX, a common and effective treatment for RA. In addition, MTX treatment of HUVEC leads to induction of cytoprotective genes such as MnSOD, and this is dependent on both AMPK and on CREB activation. This may be an important contributor to the ability of MTX to reduce CV mortality.

In order to explore this hypothesis further, a murine model of inflammatory vascular disease was bred, characterised and then treated with low-dose MTX analogous to that used to treat chronic inflammatory disease. (NZWxBXSB)F1 (WBF1) male mice develop a lupus-like illness[456], characterised by glomerulonephritis, anti-DNA and anti-phospholipid antibodies[457] and thrombocytopenia[458]. A high proportion also develop a vasculopathy affecting the gastro-intestinal tract[459] and muscular intramyocardial arteries[395], which can progress to arterial occlusion resulting in myocardial and bowel infarction. These features suggest that these mice represent a good model of a chronic immune-mediated inflammatory vascular disease in which to test whether MTX can improve outcome.

7.1.1 Lupus-prone mice

Several murine strains exist that spontaneously develop an illness analogous to SLE, characterised by a fatal immune complex-mediated glomerulonephritis and auto-antibody production. The three best known are the MRL/lpr, the (NZBxNZW)F1 and the BXSB strains. They each arose in different manners, and have phenotypic and genetic features in common and ones particular to each strain[460]. They have been closely studied for the last 40 years for the insights they yield into the genetic background and pathogenesis of SLE.

The disease in (NZBxNZW)F1 mice is more severe in female animals and its principal features are glomerulonephritis and anti-DNA antibodies[460].

The MRL/lpr strain was developed from a mixture of other strains where a recessive mutation in the Fas gene, responsible for activation of the extrinsic apoptosis pathway, arose spontaneously. In addition to glomerulonephritis and anti-DNA antibodies, these animals develop a florid necrotising vasculitis, massive lymphadenopathy and arthritis[460].

The BXSB strain is a recombinant inbred line derived from an original cross between a C57BL/6 female and SB/Le male[461]. These animals also suffer glomerulonephritis and develop anti-DNA antibodies, but also vasculitis, at a reduced frequency compared to MRL/lpr mice, splenomegaly, hypergammaglobulinaemia and hypocomplementaemia[462]. Male animals develop earlier and more

severe disease than females, with a mean survival of 5 months, compared to 15 months in females. This is due to an acceleration factor on the Y chromosome called the Y-linked autoimmune acceleration (*Yaa*) locus[463]. This region exacerbates the severity of autoimmune disease when crossed into other models, but is not enough to cause disease by itself as it is not associated with the onset of disease if crossed onto a non-autoimmune background[464].

7.1.2 WBF1 genetic background

The WBF1 model of lupus was generated by crossing the NZW female with BXSB male and was first described in 1981[456]. Like its parent strains these animals develop a lupus-like illness, with accelerated disease in male animals, although they have also been used variously as a model of acute MI, immune thrombocytopenic purpura (ITP), and anti-phospholipid antibody syndrome (APS)[458, 465, 466].

The reason disease is accelerated in this model, and in male BXSB animals, is because of the presence of the *Yaa* locus on the Y chromosome. Two papers published in 2006 established that this region is a translocation from the X chromosome containing at least 13 genes, of which the one most likely to be the accelerating factor is toll-like receptor 7 (TLR7), although TLR8 is also present[467, 468]. Male animals with a *Yaa* locus thus have a double copy of TLR7 and TLR8 and transcription of these genes is increased roughly two-fold.

TLR7 recognises single stranded RNA and is expressed by B cells, dendritic cells and macrophages. A major functional result of its ligation and activation is production of type 1 interferons[469]. Its importance in driving disease in WBF1 animals is shown by a study in which female WBF1 mice were treated with IFN α , which markedly accelerated the onset of proteinuria and death[470]. As it is known that increased circulating IFN α is an important driver of SLE activity in patients and may be important in the development of CVD in SLE[54], this aspect of the model makes it particularly good for studying ways to reduce CVD in chronic inflammation in patients.

Other features of the genetic background of WBF1 animals are less well characterised, although it clearly shares features of its parents' backgrounds. In both NZW and BXSB animals, the major histocompatibility complex (MHC) is a major source of genetic contribution to disease, and there are further contributions from multiple areas on chromosome 1 including the telomeric region, and also chromosomes 3, 4 and 10[460, 471].

7.1.3 WBF1 clinical features and course of disease

WBF1 male mice develop an inexorably progressive lupus-like disease very similar to that seen in BXSB males, with immune-complex mediated glomerulonephritis, hypocomplementaemia and autoantibodies. The first features of disease generally develop around 12 weeks and then progress rapidly, with 50% mortality at 4.5 months and 90% at 8 months. Females in contrast have 50% mortality at 11 months[456].

The most striking feature of this model when originally described was the high incidence of a degenerative coronary vascular disease associated with myocardial infarction, with approximately 80% (31/37) of animals affected at the time of death in the original description[456]. Other studies have variously reported rates of vasculopathy of 85% with accompanying MI rates of 35% at 16 weeks[395], 22% MI at 16 weeks[472] and 91% vs 56% MI in animals who were alive or dead at 51 weeks[473].

The coronary vasculopathy exhibited by male WBF1 mice is not unique to the strain. Female littermates also develop it, as do BXSB males, (NZB x NXW)F1 and MRL/l animals, albeit at a lower frequency (approximately 20 - 30%)[456, 474]. It is a disease that affects the small muscular intramyocardial arteries, leading to multiple small areas of infarcted tissue, predominantly in the ventricles. It is characterised by a sparse adventitial inflammatory cell infiltrate, rather than a vasculitis. Immunofluorescence and electron microscopy studies indicate the presence of immune complexes and thrombosis, and others have demonstrated the deposition of PAS-positive immunoglobulin and complement within the arterial wall[395, 456, 474].

The vasculopathy has also been demonstrated in the blood vessels supplying the GI tract in WBF1 mice. Mizutani *et al* showed that 61% of WBF1 males develop lesions in the arterioles of the duodenum, jejunum and ileum, consisting of subintimal fibrinoid degeneration[459].

100% of WBF1 animals develop a progressive glomerulonephritis, resulting in proteinuria and renal failure as shown by rising serum creatinine. This feature is identical to that seen in parent BXSBs. Histologically, deposits of IgG and gp70 are seen in affected glomeruli[456].

Autoantibody production in the WBF1 mouse is generally of chronic low levels rather than the very high titres seen in other strains such as the MRL/lpr. Anti-single stranded DNA antibodies and anti-thymocyte antibodies were noted in the original description, and anti-gp70 antibodies have been linked most closely with the onset of coronary vascular disease and glomerulonephritis[395, 456]. They also produce anti-cardiolipin antibodies[457], which may be important in the pathogenesis of the vascular disease[459, 475].

Haematological abnormalities in WBF1 animals include splenomegaly and hepatic sinusoidal lymphocytosis, which affected 70% of male animals in the original description of the model[456].

Later in the course of disease, thrombocytopenia becomes apparent, associated with anti-platelet antibodies and increased numbers of megakaryocytes in the bone marrow. This has led to its use in some studies as a model for ITP[458].

7.1.4 Effect of treatment of WBF1 disease course

WBF1 mice have been studied in several contexts: as a model of vascular disease in SLE, as a model of ITP or APS, or as a model of microvascular ischaemic heart disease. Studies of various treatment modalities have shed light not only on what might help in the equivalent human illnesses but also on the disease course of WBF1 animals.

Immunosuppression, unsurprisingly, has been studied in WBF1 animals. Bone marrow transplantation, anti-CD4 monoclonal antibodies, cyclophosphamide or treatment with CTLA4-Ig (which blocks T-cell co-stimulation via CD28) all prevented the onset of disease if given at around 8-10 weeks of age, before the appearance of auto-antibodies[459, 476-480]. CTLA4-Ig had no effect on disease progression if started later at around 12 weeks although bone marrow transplantation did, suggesting that this illness cannot be reversed once it has begun, without total removal of the immune system. Treatments aimed specifically at preventing B-cell activation such as anti-BAFF (belimumab) and TACI-Ig (atacept) also reduce rates of MI and nephritis and improve survival[481].

One previous study has treated WBF1 mice with MTX. Mihara *et al* gave MTX 1mg/kg orally three times weekly to 7 male WBF1 animals, from 8 to 34 weeks. They did not observe any difference in rates of proteinuria, anti-DNA antibody titres or survival between treated and control animals[482]. However, aside from the very small numbers used, they may have masked an effect by using a high dose of MTX that may have accumulated as the animals' renal function declined, causing toxicity. Their study end-point was also very long: previous reports suggest a 90% mortality at 8 months so a modest effect of MTX may have been lost by this point.

WBF1 mice have also been treated with cardiac drugs. ACE inhibition, nifedipine and bretylium improved mortality and lowered blood pressure while nifedipine and bretylium also reduced the rate of MI [465, 480, 483]. Given that thrombosis is a key histological feature that leads to tissue infarction, anti-coagulation has also been tried from the age of 2 months. Aspirin resulted in a small reduction in coronary vasculopathy and MI and delayed the onset of glomerulonephritis, but heparin and ticlopidine had no effect[480].

Finally, calorie restriction has been tested as a treatment, and found to be very effective. Mizutani *et al* compared a normal diet with a 32% reduction in available calories (from an average of 10 to 7 calories/ mouse/ day) without affecting vitamin and mineral intake, started at 6, 14, 17 or 22 weeks. Calorie restriction starting at 6 weeks resulted in 90% survival at 40 weeks, compared to 15% in animals not subjected to calorie restriction, and 60%, 35% and 20% survival in those who started calorie restriction at 14, 17 or 22 weeks respectively. All forms of calorie restriction improved the

coronary vasculopathy, glomerulonephritis and reduced titres of anti-DNA and anti-cardiolipin antibodies[484]. Although the mechanism underlying this was not explored, AMPK activation may have been important, given that it is activated by calorie restriction and has anti-inflammatory effects[266].

7.1.5 Hypothesis and aims

The aim of the work presented in the chapter was to establish and characterise WBF1 animals in our laboratory, and then treat them with low-dose MTX, with the aim of exploring the following questions:

Does MTX activate AMPK and up-regulate vascular cytoprotective gene expression *in vivo*?

Does MTX alter the course of disease in WBF1 animals?

Does MTX have functionally important anti-inflammatory effects on the vasculature of WBF1 animals?

7.2 Results

7.2.1 Characterisation of WBF1 murine vasculopathy model

The WBF1 male model of SLE and inflammatory vasculopathy was initially studied by examining cohorts of male and female animals aged 12, 16 and 24 (females only) weeks. After euthanasia, animals were studied post-mortem for signs of organ infarction and other evidence of vascular disease, and the results are shown in Table 7.1.

Female animals showed no evidence of vascular disease at any age. In male animals there was a single death at 16 weeks, and a slightly lower rate of MI than would be expected from the literature[395, 456, 472, 473]. A single bowel infarct was observed in a male animal at 16 weeks. Representative images of the myocardial infarcts seen are shown in Figure 7.1. Infarcts predominantly involved the right ventricle, and large ones were visible macroscopically (Figure 7.1 A and B). Both old infarcts resulting in fibrosis and acute infarcts with myocardial necrosis were observed (Figure 7.1 C and D).

Myocardial infarcts in this model are thought to be due to a vasculopathy of small muscular arteries, characterised by a little or no adventitial inflammatory cell infiltrate and deposition of immunoglobulin and complement causing PAS-positive staining of the vessel wall[395, 474]. Similar vascular injury has been reported involving the gastro-intestinal tract and may be responsible for the bowel infarcts observed[459]. Representative images of the vasculopathy of the intramyocardial arteries are shown in Figure 7.2.

Aortic aneurysms were found in 2 male animals at 16 weeks. These have not been reported in this model before. Both saccular and fusiform aneurysms were observed (Figure 7.3 A and B). It was very difficult to obtain histology, as the diseased aortae did not easily hold together for embedding in OCT, but frozen sections through a fusiform aneurysm were obtained and an unstained transverse section is shown in Figure 7.3 C. In this case a dissection had developed - the fraying of the aortic wall at the intima-media junction is visible, and the false lumen is filled with blood.

Finally, hepatosplenomegaly was observed in both 12 and 16 week males, but more frequently in the older cohort. This was not seen in any 12 or 16 week old females, but in 2/6 24 week females.

	Male		Female		
	12 weeks	16 weeks	12 weeks	16 weeks	24 weeks
Numbers	11	13	6	6	7
Deaths	1	1	0	0	0
Myocardial infarct	2	3	0	0	0
Aneurysm	0	2	0	0	0
Bowel infarct	0	1	0	0	0

Table 7.1 Characteristics of (NZWxBXSB)F1 murine vasculopathy model.

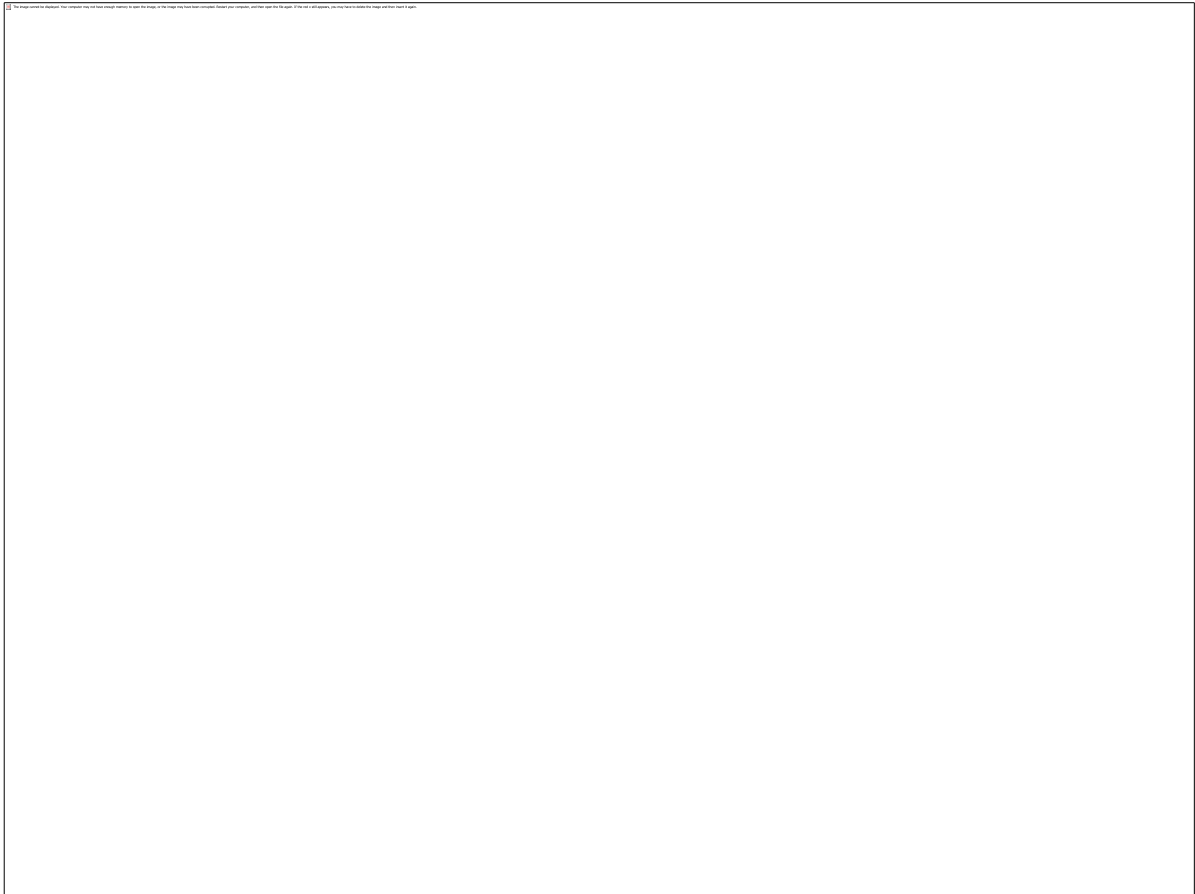


Figure 7.1 WBF1 myocardial infarcts. A: the macroscopic appearance of a WBF1 heart with a large right ventricular infarct. B: x10 magnification image of a short axis section taken at the mid-ventricular point showing a large haemorrhagic right ventricular infarct. C: x40 magnification image of an old, fibrotic right ventricular infarct. D: x40 magnification image of an acute, haemorrhagic right ventricular infarct.

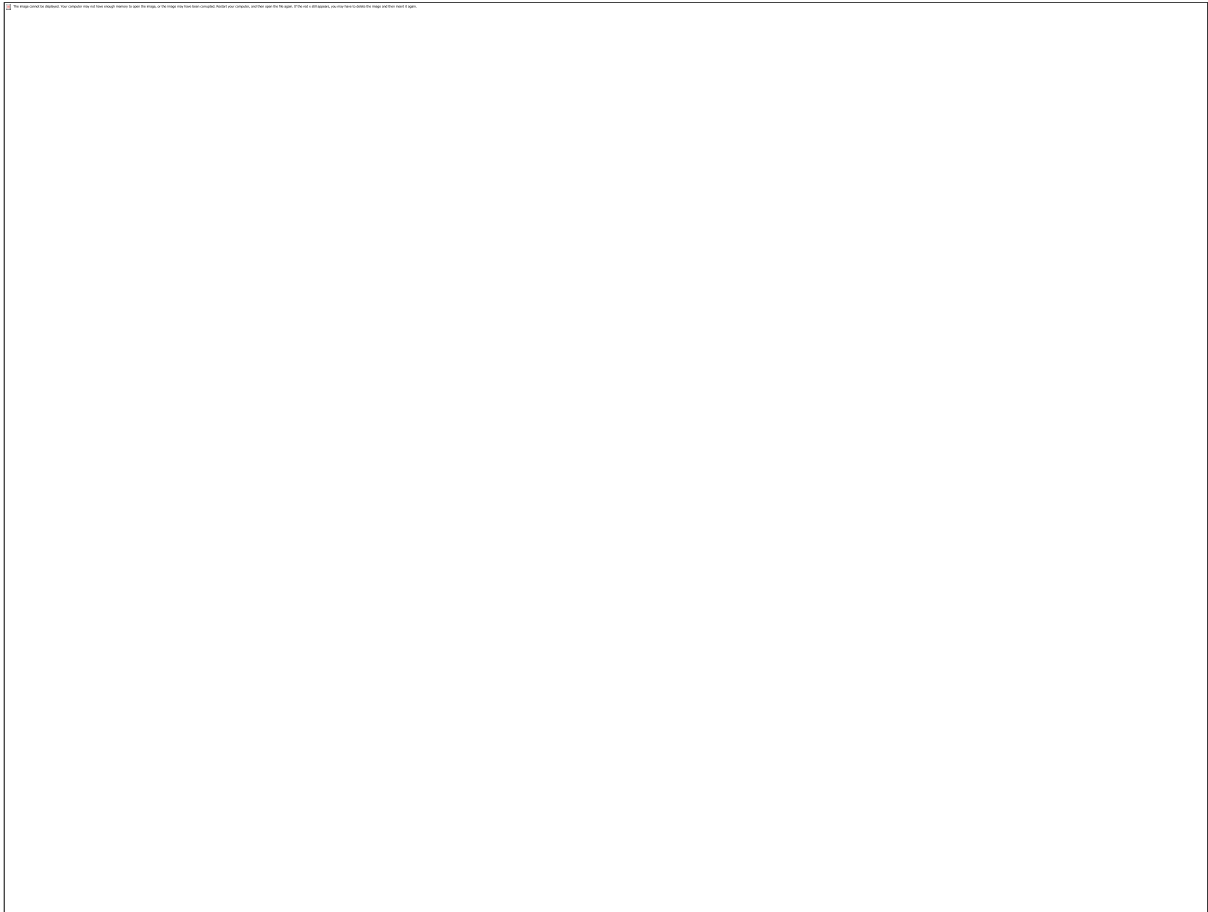


Figure 7.2 WBF1 vasculopathy of the intramyocardial arteries. A: photomicrograph of an intramyocardial artery demonstrating the mild adventitial leucocytic infiltrate (arrows). H&E stain, x200 magnification. B and D: same vessel as shown in A at x100 and x40 magnification respectively. C: PAS staining of same vessel, x200 magnification. These photomicrographs were taken by Dr JJ Boyle.

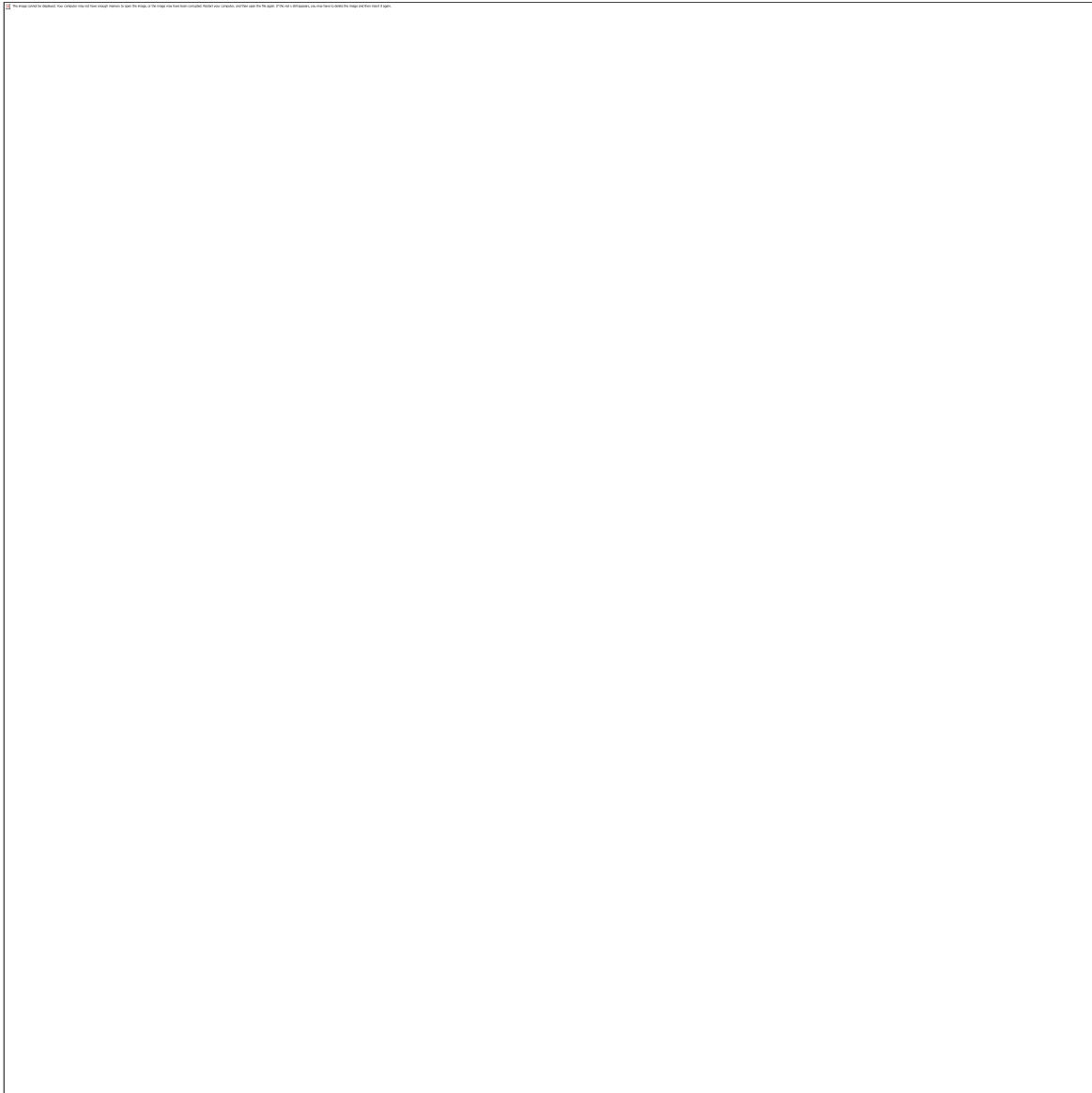


Figure 7.3 WBF1 aortic aneurysms. A: a saccular aneurysm of the descending aorta. B: a fusiform aneurysm or dissection of the descending aorta. C: low power, unstained frozen section of a dissected aorta.

7.2.2 MTX increases vascular MnSOD expression *in vivo*

WBF1 male mice were treated with MTX 1mg/kg or 0.9% saline by weekly intraperitoneal injection for 4 weeks from 12 to 16 weeks of age. The dose of MTX chosen has been shown to increase adenosine levels [376] and to protect against collagen-induced arthritis [393], and is representative of the long-term low-dose MTX therapy used to treat inflammatory diseases clinically. Treatment started at 12 weeks because the disease is known to be under way at that point[478], and the aim was to treat in a manner close to that used in clinical practice: after the onset of disease, to look for amelioration in disease features rather than prevention of its onset.

In order to determine if the changes in cytoprotective enzymes demonstrated *in vitro* also occurred when MTX is used *in vivo*, snap-frozen descending aortae harvested from the MTX- and saline-treated mice described above were analysed. Immunoblotting of aortic lysates revealed increased MnSOD protein expression in those animals treated with MTX (Figure 7.4). Female littermate controls were also analysed as a disease-free control group, and there was no significant difference in MnSOD protein expression between male and female animals.

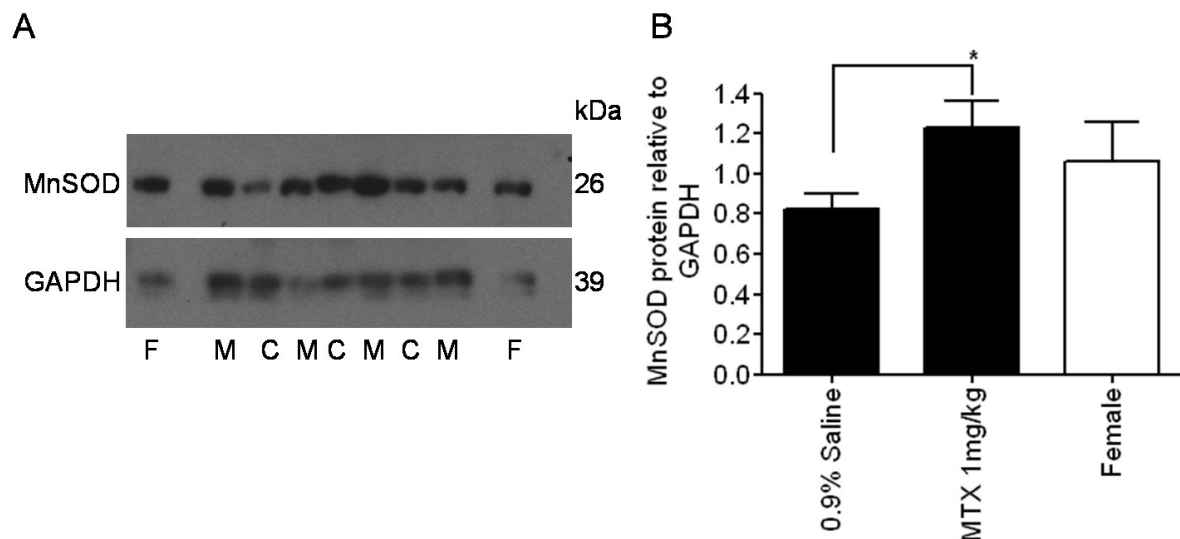


Figure 7.4 MTX increases vascular MnSOD expression in WBF1 mice. WBF1 male mice were treated with MTX 1mg/kg weekly by intraperitoneal injection for 4 weeks or 0.9% saline as control. Female littermates were used as a healthy control population. Snap frozen aortic tissue from each animal was pulverised and run in western blots. A: representative immunoblot showing MnSOD expression from 2 female, 4 MTX-treated and 3 saline-treated males; B: densitometry of MnSOD protein expression relative to GAPDH. N=18 in the MTX and control groups; and 6 females. F: female; M: MTX-treated male animal; C: male saline control animal.

*=p<0.05.

7.2.3 MTX increases AMPK phosphorylation *in vivo*

Aortic lysates harvested from MTX- and saline-treated WBF1 mice were also analysed to look for changes in phosphorylated AMPK. MTX treated animals had significantly more phosphorylated AMPK in vascular tissue (Figure 7.5). This supports the *in vitro* findings shown in Chapter 5, and suggests that enhanced AMPK phosphorylation may be a real phenomenon in patients treated with MTX.

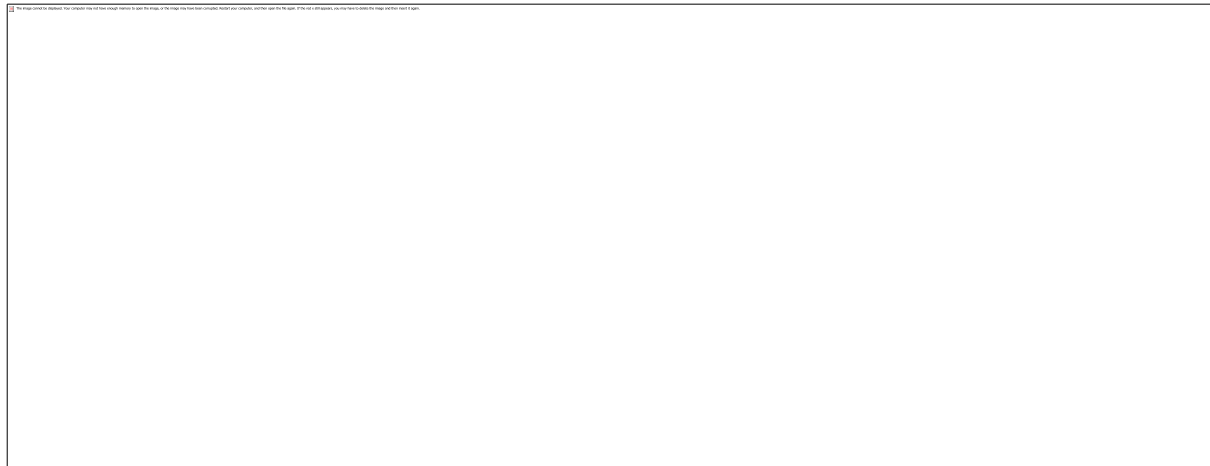


Figure 7.5 MTX increases vascular AMPK phosphorylation in WBF1 mice. WBF1 male mice were treated with MTX 1mg/kg or 0.9% saline weekly by intraperitoneal injection for 4 weeks. Snap frozen aortic tissue was ground and used in western blots. A: representative blot showing AMPK phosphorylation in 4 control and 3 MTX treated animals. B: densitometry of phAMPK expression, relative to total AMPK and GAPDH as loading control. Data shown are the results of 12 MTX treated and 10 control animals and * = $p < 0.05$. C: control animal; M: MTX treated animal.

7.2.4 MTX reduces organ damage in WBF1 mice

Besides verifying the *in vitro* findings of AMPK activation and induction of cytoprotective genes with MTX treatment of EC, a further aim of treating WBF1 mice with MTX was to establish whether it had a disease-modifying effect on the vascular disease suffered by these animals. Observation of the animals at 16 weeks, just prior to euthanasia, revealed that those treated with MTX appeared to be in better general health compared to the control. Saline-treated animals looked unwell, with ruffled fur, and were huddled together. The MTX-treated animals had smooth sleek coats and were active and inquisitive (Figure 7.6).

To establish the impact of therapeutic intervention on the systemic disease, a multisystem organ damage score was composed, based on pathology observed post-mortem (Table 7.2). This was devised according to the post mortem disease features first observed in the 16 week old male animals used to characterise the model, that were not present in their healthy female littermates. The presence or absence of hepatosplenomegaly, bowel infarcts or ascites or pleural fluid was recorded at post mortem. The presence of aortic aneurysms was recorded following microscopic dissection of the descending aortae. Finally the presence and size of myocardial infarction was examined histologically, with confirmation from a second blinded investigator, as described in section 3.3.8. The damage score revealed a significant disease-modifying effect of MTX, with no treated animals scoring 4 or 5, and a majority showing no visible signs of ill-health at all (Figure 7.7).

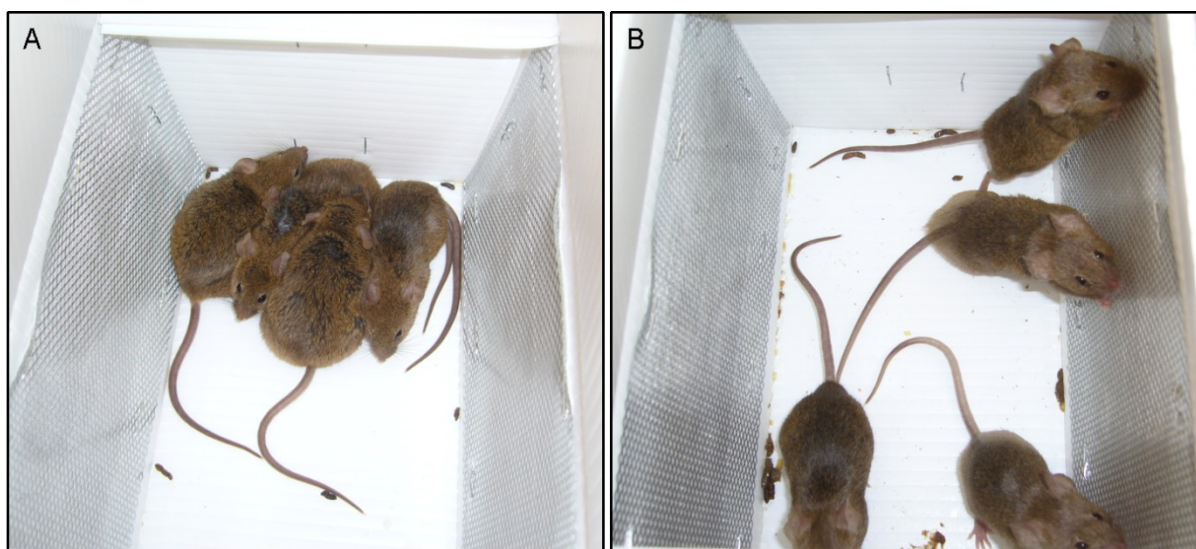


Figure 7.6 WBF1 male mice at 16 weeks. A: animals given 0.9% saline; B: animals treated with MTX.

Feature	Score
Death	2
Myocardial infarct: full thickness	2
Myocardial infarct: small fibrotic	1
Bowel Infarct	1
Aortic aneurysm	1
Ascites or pleural fluid	1
Hepatosplenomegaly	1

Table 7.2 Composition of WBF1 post mortem damage score

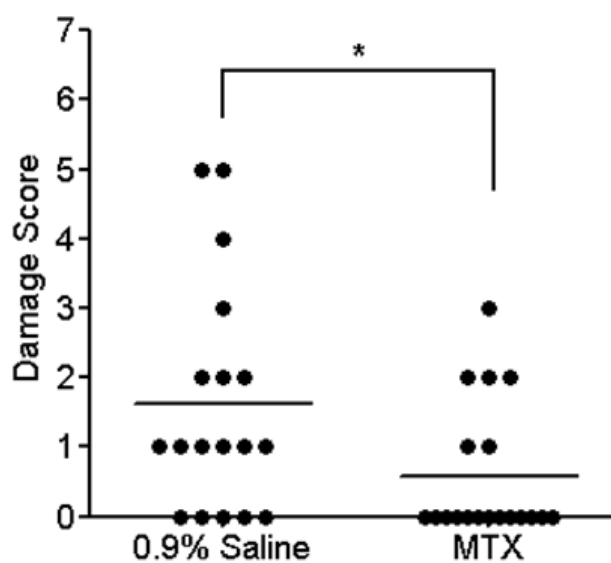


Figure 7.7 MTX improves damage score in WBF1 mice. A damage score was constructed (Table 7.2), based on post mortem finding in the original characterisation cohort, and applied to post mortem findings in all mice treated with MTX or 0.9% saline. Data shown represent the results from 18 control and 19 MTX-treated animals. * = $p < 0.05$.

7.2.5 MTX improves the vasculopathy of intramyocardial arteries

Having established that MTX ameliorated the WBF1 disease in general, its effect on the coronary vasculopathy was investigated. PAS-stained myocardial sections were examined by myself and a second investigator (with thanks to Dr JJ Boyle) and a modified scoring system based on Berden *et al* was formulated[395]. This involved quantifying the vasculopathy in two ways: firstly, by counting infiltrating leukocytes in the adventitia; and secondly by scoring the deposition of PAS-positive material in the arterial walls. The scoring was as follows: 0: normal staining; 1: increased basement membrane staining; 2: increased basement membrane staining and nodular deposits. A single section per animal was examined, each of which contained approximately 10-15 intramyocardial small arteries.

Following four weeks' treatment with MTX, a significant reduction in the vasculopathy affecting the intramyocardial arteries was observed, with MTX treatment causing a reduction in both the numbers of adventitial leukocytes and the degree of PAS staining of the vessel walls (Figure 7.8). This provides direct evidence that low-dose MTX can improve an inflammatory vascular disease.



Figure 7.8 MTX improves WBF1 coronary vasculopathy. Myocardial sections stained with PAS from WBF1 mice treated with MTX 1mg/kg (n=19) or normal saline (n=17) weekly from 12 - 16 weeks were examined and the vasculopathy quantified. Firstly, the number of leukocytes infiltrating the adventitia was counted. Secondly, the deposition of PAS-positive material in the arterial walls was scored. A: an artery with a large number of infiltrating leukocytes; B: an example of heavy deposition of PAS-positive material; C and D: examples of normal arteries with no leukocyte infiltration and no excess PAS staining; E: quantification of adventitial leukocyte infiltration; F: quantification of PAS staining of arterial wall. AU: arbitrary units. * = $p < 0.05$; *** = $p < 0.001$.

7.2.6 Effect of MTX treatment on myocardial infarction in WBF1 mice

Having established that low-dose MTX attenuates the histopathological appearance of the arterial vasculopathy in WBF1 mice, I next studied whether the reduction in vascular disease was associated with a reduction in myocardial infarction. Coded myocardial sections were examined by two investigators including me (with thanks to Dr JJ Boyle) to establish which animals had suffered myocardial infarctions. At 16 weeks, 7/17 (41%) untreated mice had suffered an MI and 4/19 (21%) MTX-treated animals. Although this represents a near 50% reduction in MI rate, the proportion of affected animals is too small for the difference to be statistically significant. However, the rate is consistent with previous reports of MI incidence in this model at this time point[395, 472]. The optimal time point to study for an effect on MI is probably 24-30 weeks when the incidence is 53-62%, but the data would be likely to be confounded by the onset of renal impairment and thus increased MTX toxicity.

Myocardial damage was quantified in two ways. Firstly serum cardiac troponin I (cTnI), a highly sensitive and specific marker for myocardial damage, was measured in post-mortem blood samples by ELISA. Although large troponin rises were seen only in untreated animals, no statistically significant effect of MTX treatment was observed (Figure 7.9). This was because the majority of animals in each group did not have detectable cTnI levels, including animals in which MI was seen histologically. This reflects the time course of cTnI release following MI: as it is a relatively acute marker and cleared within two weeks in the majority of cases, animals with older MIs were unlikely still to have detectable serum cTnI.

Secondly, infarct area was quantified on sections stained with picrosirius red. This is a specific stain for collagen and therefore can be used to show fibrotic damage caused by prior infarction. Areas of infarct were photographed consecutively and the images quantified on the green channel in Image J by thresholding using the histogram function. The area of the image that stained red and thus constituted infarct was obtained, and this was expressed as a proportion of the area of the whole myocardial section (obtained from separate ultra-low power photographs). However, given that a small number of animals had suffered acute MI that had not yet been converted to collagen scar tissue, using this method alone would have under-reported the extent of MI. Therefore acute MI, as identified by two investigators, was also photographed and quantified by straightforward area measurement in Image J. However, although fewer animals treated with MTX suffered an MI, and those that did had small infarcts, no statistically significant difference in myocardial area could be demonstrated between the groups (Figure 7.10).

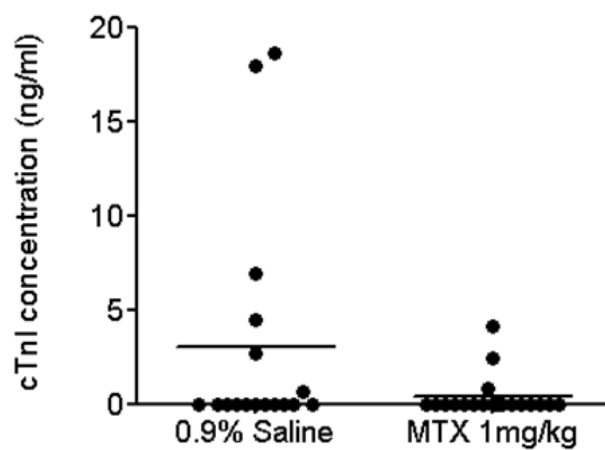


Figure 7.9 Serum cTnI concentration in WBF1 mice treated with MTX or saline control. Murine serum cTnI was quantified by ELISA. N=17 controls and 19 methotrexate treated animals. Data are expressed as mean and were analysed by Mann-Whitney U-test. No significant difference between the groups was observed.



Figure 7.10 Quantification of MI area using picosirius red staining. All photomicrographs are of myocardium stained with picosirius red and taken at x40 magnification. A: an extensive fibrotic infarct involving the entire right ventricle; collagen stained red. B: infarcts of various ages are seen in a single animal: an acute infarct staining dark brown in the free wall of the right ventricle, and an old fibrotic infarct staining red in the septal wall of the right ventricle. C: a large acute infarct involving the free wall of right ventricle. D: a small fibrotic infarct in an animal treated with MTX. E: quantification of infarct size; ns: not statistically significant.

7.2.7 MTX reduces aortic endothelial and adventitial ICAM-1 expression

The final question addressed was whether MTX exerts an anti-inflammatory effect on the vasculature of WBF1 mice. To do this, aortic ICAM-1 expression was studied. Transverse sections of descending aortae from MTX and saline-treated WBF1 mice and from a cohort of female littermates as healthy controls were stained with an anti-mouse ICAM-1 mAb, anti-CD31 and DRAQ5 and examined by immunofluorescence confocal microscopy (a single section per animal). Male animals had significantly greater aortic endothelial and adventitial ICAM-1 expression than females (Figure 7.11), reflecting the presence of inflammatory vascular disease in the male study population. MTX treatment significantly reduced endothelial and adventitial ICAM-1 staining (Figure 7.11). The reduction in adventitial staining is likely to be of particular relevance in the amelioration of organ damage in this model, given the reduction in the adventitial leukocyte inflammatory infiltrate also observed in the intra-myocardial arteries (Figure 7.8).

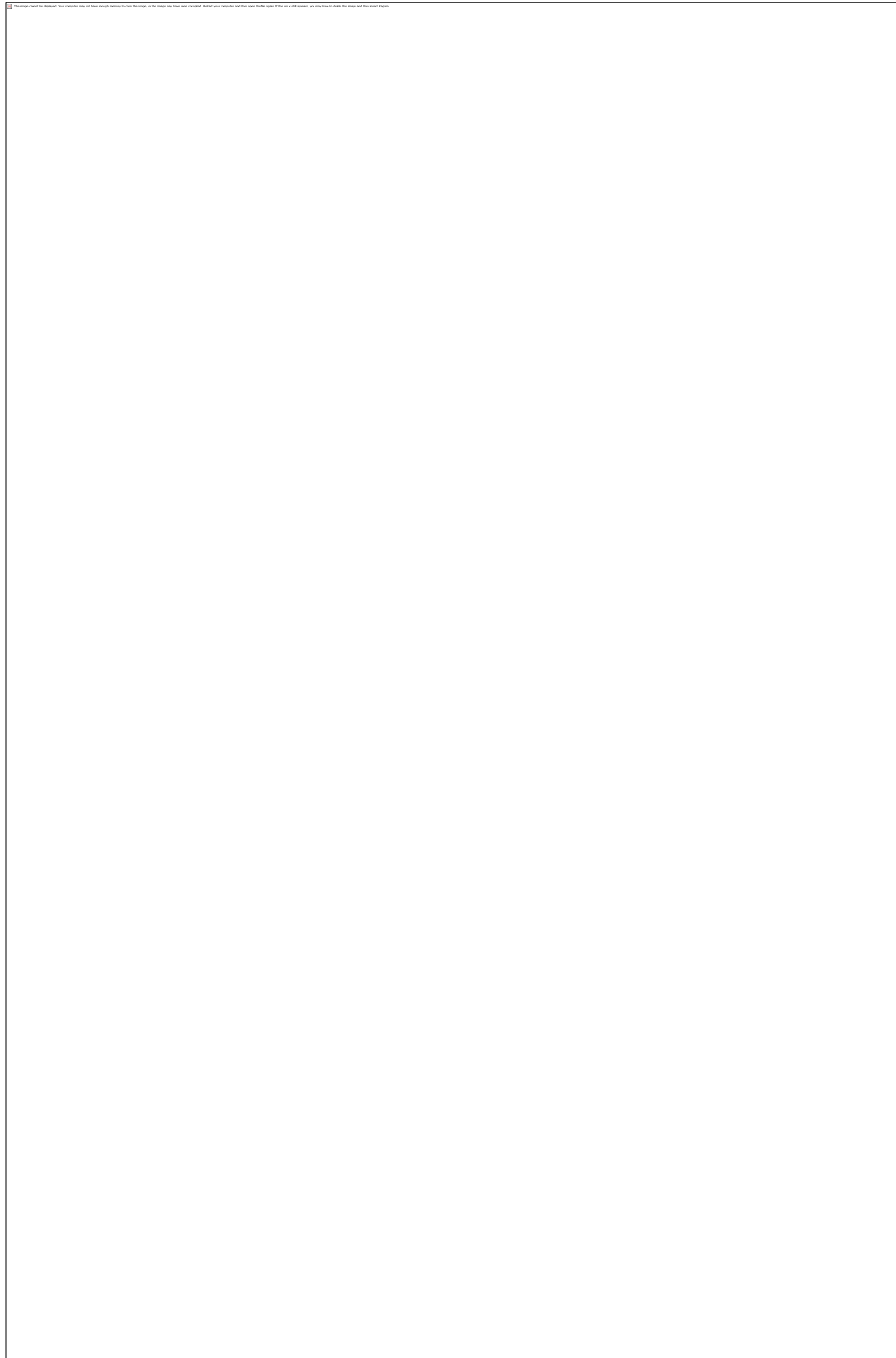


Figure 7.11 MTX reduces aortic endothelial and adventitial ICAM-1 expression in WBF1 male mice. WBF1 male mice were treated with MTX 1mg/kg weekly for four weeks. Frozen transverse sections of descending aorta were stained with anti-ICAM-1 and mean fluorescence intensity was measured. A: control WBF1 male; B: MTX-treated WBF1 male; C: female littermate control; D: quantification of endothelial fluorescence; E: quantification of adventitial fluorescence. Confocal microscopy images: green: anti-CD31; red: anti-ICAM-1; purple: DRAQ5 nuclear stain. Data are expressed as mean $\pm$ SEM. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

7.3 Discussion

This chapter presented experiments exploring the effects of using MTX to treat male WBF1 mice, which develop a lupus-like illness and an inflammatory vasculopathy of small and medium-sized arteries that leads to thrombosis and tissue infarction. The model was first established in the laboratory, and then a cohort of animals were treated with MTX 1mg/kg weekly by intra-peritoneal injection for 4 weeks from 12 - 16 weeks of age, and compared both to littermate controls treated with 0.9% saline and to healthy female siblings. MTX treatment resulted in increased expression of phosphorylated AMPK and MnSOD in aortic lysates, and in addition reduced aortic adventitial and endothelial ICAM-1 expression providing direct evidence of an anti-inflammatory effect. This was reflected by reduced organ damage and improved coronary vasculopathy, but there were not sufficient animals affected to show an effect on myocardial infarction.

7.3.1 Choice of murine model

Any animal model of atherosclerosis will only ever be an approximation; the natural course of disease takes decades and so cannot really be replicated over the working lifetime of an interested researcher. In the context of chronic systemic inflammation, rodent models of RA such as collagen-induced or adjuvant arthritis only last 4 -6 weeks and thus are unlikely to have the time to develop significant vascular complications. Lupus-prone mice have longer lasting disease (several months) but develop degenerative vascular disease in only around 20% of animals, with the exception of the WBF1 model used here[474].

Most previous *in vivo* studies of CVD in SLE or RA and CVD have done so by crossing strains of lupus prone or arthritis-susceptible mice with Apo E^{-/-} mice. Apo E^{-/-} mice have grossly deranged plasma lipids with low HDL and high total cholesterol and LDL and spontaneously develop cholesterol-laden atheromata. Attempts have been made to study the links between RA and CVD using CIA-susceptible, Apo E^{-/-} mice. These animals developed more severe arthritis, but no change in the severity of atherosclerosis was observed[485].

To study CVD in the context of SLE. Aprahamian *et al* used *Gld.apoE^{-/-}* animals, deficient in both Fas ligand and Apo E as their model to study whether rosiglitazone could ameliorate disease manifestations[486]. Others have used Apo E^{-/-} mice crossed with Type I IFN receptor deficient mice, or looked at cardiometabolic risk profile or markers of endothelial dysfunction in standard lupus-prone mouse models[55, 487, 488].

The principal problem with all these models is that they either involve measuring indirect surrogate markers for vascular disease, or involve using a model with such gross derangement of lipid profile that extrapolation to the human situation is difficult. In addition Apo E^{-/-} mice, although they develop extensive atheromata, very rarely suffer plaque rupture and vessel occlusion.

The WBF1 model that I used is not a model of atherosclerosis but rather of an inflammatory vasculopathy in which it is likely that anti-phospholipid antibodies play a pathogenic role. However, the vasculopathy is frequently associated with a local inflammatory cell infiltrate, vessel thrombosis and occlusion, causing tissue infarction, and thus I felt it was a closer approximation to the vascular injury associated with SLE than any other model.

7.3.2 Characterisation of the WBF1 model

The initial work presented in this chapter was the original characterisation of the WBF1 model that I performed. Cohorts of male and female animals aged 12, 16 and 24 weeks were studied for signs of vascular disease: myocardial and bowel infarcts and aortic aneurysms. As expected, female animals showed no signs of disease even at 24 weeks, while male animals were affected in low numbers at 12 weeks and slightly more at 16 weeks. Overall, however, the numbers of affected animals appeared lower than the original descriptions of the model suggested, with a smaller proportion suffering MI or death[456]. Although the coronary vasculopathy was present in a high proportion of animals, the appearances seemed less florid. In particular, large deposits of PAS-positive material in vessel walls were very rarely observed, and this meant that the scoring system devised by Berden *et al*[395] had to be modified when I quantified the disease. It is not clear why this would be, but environmental influences are likely to be important in modulating the onset and severity of disease, and there are no doubt differences in pathogen exposure, diet and bedding material between the conditions the animals used in these experiments were housed in and those of the animals in the original reports.

In addition, I noted aortic disease in a small number of older male animals both in the initial characterisation experiments and in those used to study the effects of MTX. Macroscopically these appeared to be fusiform and saccular aneurysms, in some cases initiating near branch points where intercostal arteries originated. However, although gaining histological sections was extremely difficult due to the fragile nature of the tissue, frozen sections through one indicated that it was in fact an arterial dissection. It was not possible to study the pathology underlying this aortic disease, but it is known that the WBF1 mouse is hypertensive so this could be contributing[480]. Vasculopathy affecting the vasa vasorum might also be present.

7.3.3 MTX treatment of WBF1

The arterioprotective actions of MTX were explored by treating WBF1 mice with a low dose, once disease was established but still early in its course. The intervention was timed to model the treatment of early systemic inflammatory disease in patients, and aimed to demonstrate amelioration of the vasculopathy and a reduction in end-organ damage due to infarction. Therefore, they were given MTX 1mg/kg weekly for 4 weeks from 12 to 16 weeks. At 12 weeks disease is detectable in WBF1 mice[395, 478, 481], while by 16 weeks over 80% would be expected to have developed the

vasculopathy and around 40% to have suffered an MI[395]. Thus this seemed a good time point to look for disease modification, without causing suffering to a large proportion of animals.

The dose of MTX was chosen to be as low as possible while still exerting a pharmacological effect. It was chosen on the basis of that shown to alter adenosine levels *in vivo*, and that shown to be effective in treating rodent models of inflammatory arthritis[376, 393, 394]. 1mg/kg is higher than the doses used to treat inflammatory arthritis in adults (typically 15-25mg weekly), but not dissimilar to those used in juvenile idiopathic arthritis, where doses up to 30mg in 30 - 40kg children can be used.

A previous attempt to treat this model with MTX failed to show any improvement in mortality or renal function at 34 weeks[482]. However, this study used three-fold higher doses of MTX, which may have contributed to mortality given that MTX accumulates in renal failure, and the end-point was a late stage in an unremitting disease where a large percentage of animals would already be expected to have died. I studied an early time point, in the hope that this meant disease might be more easily modulated, rather than waiting for irreversible damage to set in. This also reflects current clinical practice, where we aim to treat as early as possible in the course of disease.

Using low dose MTX in WBF1 mice resulted in increased vascular expression of phosphorylated AMPK α and MnSOD, replicating the major *in vitro* findings reported in Chapters 5 and 6. These changes were observed in lysates prepared from whole aortae and thus represent changes in all cells within the vessel wall, of which EC are only a small proportion. However, this increases the likelihood that these changes might also occur in patients treated with MTX, as they have now been observed in both *in vitro* cultures of primary human cells and in an animal model of chronic inflammatory disease, and at doses compatible with those used in clinical practice.

7.3.4 WBF1 disease modification with MTX

Treating WBF1 mice at an early stage in disease with MTX attenuated organ damage and reduced the severity of coronary vasculopathy with a reduction in both infiltrating adventitial leucocytes and in PAS-positive material in vessel walls. Although fewer animals treated with MTX suffered an MI, and those that did suffered smaller infarcts than controls, there were not enough affected animals for this difference to be statistically significant. There were a few technical limitations however that may have contributed. Measuring cTnI captures myocardial damage at a single time point; the levels fall away over a few days after myocardial damage occurs. Using it to quantify MI on post mortem blood samples will thus have missed infarcts more than a few days old. Conversely, quantifying infarcted tissue histologically was so time consuming that only a single slide cut at the mid-ventricular point from each animal could be analysed. Although it had the advantage of capturing both old and new infarcts, small lesions elsewhere in the heart will have been missed. Thus both methods used may have underestimated the number of MIs suffered by the cohort.

However, the multisystem organ damage score did show an effect of MTX on disease progression in this model. This too however is subject to some limitations. As I treated animals with MTX only once, it was not possible to validate the score in advance on a group of treated animals: instead it was composed of those disease features apparent in 16 week old male mice but not in their healthy female littermates when I first characterised the model. Secondly, there is a concern about bias given that I both treated the animals and carried out the post mortem examination. Although I used a coding system to blind myself regarding treatment condition, this will not have been perfect given that I knew from which cage each animal I culled came from. However, the myocardial sections were examined by second investigator who was blinded, and we agreed on the presence or absence of myocardial infarcts and of the vasculopathy together.

MTX also exerted a specific anti-inflammatory action, reducing expression of aortic endothelial and adventitial ICAM-1. The reduction in adventitial ICAM-1 may be of relevance to the mechanism by which MTX improved the coronary vasculopathy, as it may have contributed to the reduction in adventitial leucocyte infiltration. A reduction in endothelial ICAM-1 is an important observation in terms of how MTX retards atherogenesis in general, as increased expression of EC adhesion molecules is a central feature of endothelial dysfunction and of critical importance for the recruitment of leucocytes into atherosclerotic plaques. MTX has previously been shown to reduce soluble ICAM-1 in blood samples from patients with early RA and with heart failure, as well as surface ICAM-1 on HUVEC *in vitro* [382, 431, 489]. However, I did not find that treating HUVEC with MTX reduced the induction of ICAM-1 stimulated by TNF α (Figure 5.10). This discrepancy between *in vitro* and *in vivo* findings illustrates the importance of doing both in order to be confident whether the results are meaningful.

I have not been able to show whether AMPK activation and increased expression of downstream cytoprotective genes were necessary for the protective effects of MTX in this model. This could have been done by, for example, treating animals with an AMPK inhibitor such as Compound C in addition to MTX. Unfortunately time constraints did not allow me to investigate this.

The experiments presented in this chapter have demonstrated therefore that MTX can exert arterioprotective effects *in vivo*, increasing vascular AMPK activation and MnSOD expression and reducing ICAM-1. These changes may be important for its ability to modulate the course of a murine inflammatory vasculopathy.

8 CONCLUDING REMARKS

8.1 Introduction

Premature CVD complicates chronic systemic inflammatory disease such as RA and SLE[12]. Understanding of the mechanisms that underlie this has progressed but remains incomplete. As a result, although treatment for these conditions has improved radically in the last 30 years, we lack knowledge of how to prevent or retard the associated vascular injury.

Atherosclerosis begins with endothelial dysfunction, a state characterised by reduced bioavailability of NO, increased expression of cell adhesion molecules and oxidative stress[91]. Endothelial dysfunction is clinically detectable by non-invasive means and is reversible whereas established atherosclerosis is not[90]. If it were known therefore which drugs, or drug combinations, were best able to reverse endothelial dysfunction, it might be possible to construct a pharmacological strategy to tackle CVD in chronic inflammation.

Normal endothelium exists in a quiescent state, with low cell turnover and energy use, a reducing intracellular environment and tonic production of NO[11]. This state is promoted by high flow LSS and, in some vascular beds, VEGF. If the balance is disturbed by oxidative, inflammatory or metabolic insults, cytoprotective pathways operate to counteract these and restore homeostasis. These include induction of anti-oxidant enzymes such as MnSOD and HO-1 and anti-apoptotic proteins such as Bcl-2 and A1, modulation of mitochondrial metabolism via uncoupling proteins, and enhanced protection against complement-mediated injury via DAF.

One signalling kinase that is capable of responding to cellular stressors is AMPK. AMPK is classically activated by metabolic or nutritional stress, and the outcome is conservation of, or enhanced efficiency in the use of, cellular energy[261]. In EC, AMPK activation increases NO production, protects against oxidative injury and prevents apoptosis[142]. It also has effects on mitochondrial metabolism by promoting mitochondrial biogenesis[310]. AMPK can be activated by several classes of drugs that have vasculoprotective effects, including statins, metformin and fibrates, but these observations are limited largely to *in vitro* studies using drug concentrations several fold higher than those achievable in patients.

MTX, an anti-inflammatory drug central to the current treatment paradigms for RA, is probably prescribed long-term for several hundred thousand people in the UK (by my own estimate). Robust evidence shows that its use is associated with a reduction in risk of CVD in RA[79]. Despite such widespread use, its anti-inflammatory mechanism of action is not completely understood. The best evidence suggests that MTX acts by inhibiting enzymes involved in nucleotide metabolism, resulting in increased adenosine levels[369]. However, other nucleotides whose intracellular levels are also

altered by MTX include AICAR, ZMP and AMP, all of which are capable of allosteric activation of AMPK.

Thus, the hypothesis I formulated was that MTX acts to protect vascular endothelium against inflammatory injury by activating AMPK, and that this leads to enhanced expression of cytoprotective genes. If present, such a mechanism might explain why the use of MTX in RA is associated with reduced CV mortality, and may provide a pharmacological strategy for the prevention of atherosclerosis in chronic systemic inflammation.

8.2 Research questions

The work presented in this thesis sought to explore mechanisms through which endothelial cytoprotective pathways might be activated, in order to offer pharmacological protection against vascular injury to patients with chronic inflammatory disease. Four questions were addressed:

The endogenous mediators of endothelial cytoprotection VEGF and high flow LSS regulate many similar pathways in EC. Do confluent EC conditioned by LSS react differently to VEGF compared to static-cultured cells, resulting in a synergistic induction of cytoprotective pathways?

Does MTX activate AMPK and enhance cytoprotective gene expression in EC, and does this have a functional benefit?

If it occurs, by what mechanisms does activation of AMPK by MTX lead to changes in gene expression?

Does MTX activate AMPK and enhance cytoprotective gene expression *in vivo*, and can it improve outcome in a murine model of inflammatory vascular disease?

8.3 Major findings

8.3.1 EC responses to VEGF when conditioned by high flow LSS are generally not synergistic

Confluent HUVEC were exposed to high flow LSS using the Cytodyne streamer parallel plate apparatus for 16 - 24h, before the addition of VEGF 25ng/ml for periods ranging from 4 to 24h. Previous reports had indicated that both VEGF and LSS could induce endothelial expression of the cytoprotective enzyme HO-1 and so I looked for a synergistic induction. However, although LSS strongly induced HO-1, VEGF did not under static conditions, and a synergistic increase was observed only with mRNA, and transiently at a short time point only.

Synergy was not observed with other proteins whose expression is thought to be regulated by both VEGF and LSS such as VEGFR2 and eNOS, while little difference in expression was observed with DAF, known to be regulated by VEGF but not LSS. An intriguing effect was noted with the anti-apoptotic protein A1, whose expression is regulated by VEGF. LSS for 24h strikingly down-regulated expression and VEGF added to LSS-exposed cells was able to overcome this and strongly induce expression. However, this was a transient phenomenon that had recovered by later time points and was probably an artefact related to the process of endothelial equilibration to flow.

Finally, a phosphokinase array was performed to explore differences in phosphorylation status in HUVEC treated with VEGF for 15 minutes after 24h equilibration to high flow LSS. Although VEGF was still able to enhance phosphorylation of a small number of kinases, notably AMPK, JNK and p27, in most cases kinases that were phosphorylated by VEGF in static conditions or by LSS were dephosphorylated when VEGF was added to EC conditioned to LSS. This may reflect homeostatic mechanisms, where the effect of two strong drivers of a phenomenon acting together is a reduction in its magnitude. Another possible explanation is that VEGF is a growth factor and endothelial mitogen, while LSS generally promotes a quiescent state with low cell turnover and thus the two together may act in opposition. What is clear, however, is that in general EC responses to VEGF when conditioned by high flow LSS are not enhanced compared to those in static culture.

8.3.2 MTX activates AMPK and induces cytoprotective gene expression in EC, resulting in enhanced protection against apoptosis

One of the few kinases whose phosphorylation state was enhanced by VEGF in HUVEC conditioned by LSS was AMPK, and this kinase formed the basis for investigating pharmacological approaches to enhance endothelial cytoprotection in patients suffering chronic inflammatory diseases.

AMPK α^{Thr172} phosphorylation was increased by treating HUVEC with MTX 100nM for 48h. This concentration is close to those achieved therapeutically in patients. Cytoprotective gene expression was also observed when both 100nM and 10nM MTX were studied; with induction of MnSOD and

HO-1 protein and mRNA, and also DAF, A1 and UCP-2 mRNA. Induction of MnSOD protein was also demonstrated in HAEC. These changes did not represent a general response to an oxidative insult as no change was seen in expression levels of Trx, Trx reductase or NQO-1. Induction of MnSOD and HO-1 were preserved if HUVEC were co-treated with folic acid and MTX, and if a dysfunctional endothelium was modelled by pre-treatment with TNF α . MTX treatment did not alter the induction of cell adhesion molecules by TNF α , but did protect against apoptosis induced by glucose deprivation. Consistent with its known actions in inhibiting folic acid metabolism however, it also reduced EC proliferation.

These results suggest that long exposure to MTX at therapeutically relevant concentrations exerts a beneficial effect on HUVEC by activating AMPK and inducing cytoprotective gene expression. As the responses are preserved in the presence of folic acid and TNF α , they may be of relevance to the mechanism of action in patients prescribed MTX.

8.3.3 MnSOD and HO-1 induction of MTX is dependent on AMPK and CREB

Although I had demonstrated that MTX activates AMPK in HUVEC and enhances expression of cytoprotective genes, whether the changes in gene expression were the result of AMPK activation had not been proved. Furthermore the transcription factors involved had not been identified.

Two different loss-of-function approaches were employed to establish the role of AMPK in the induction of MnSOD and HO-1 by MTX. First, AMPK was inhibited using Compound C. This reduced the induction of MnSOD and HO-1 mRNA by MTX but not protein; in fact Compound C itself induced MnSOD and HO-1 protein and this probably masked any effect of MTX via AMPK. Therefore, RNA interference was used to deplete AMPK α 1 and/ or AMPK α 2. Using this technique it was established that inhibiting AMPK α 2 signalling prevents MTX inducing MnSOD and HO-1 and inhibiting AMPK α 1 prevents the induction of HO-1 and may also affect MnSOD. As a further step, HUVEC were treated with the AMPK activator AICAR and induction of MnSOD was observed.

Previous reports indicated that a good candidate for the transcription factor involved in mediating induction of MnSOD by AMPK activation was CREB, as this is a direct target of AMPK and it has previously been demonstrated to mediate MnSOD expression in cancer cells in response to TPA[200, 445]. MTX 100nM for 48h resulted in increased CREB^{Ser133} phosphorylation, and this was inhibited in HUVEC treated with AMPK α 1 or AMPK α 2 siRNA, indicating that CREB lies downstream of AMPK. CREB depletion by RNA interference abolished the induction of MnSOD and HO-1 by MTX, and ChIP demonstrated enhanced binding of CREB, most probably at a known, validated, CRE site, to the SOD2 promoter.

Finally, the role of ROS in the induction of HO-1 and MnSOD by MTX was investigated. MTX itself caused a small rise in intracellular ROS. Pre-treatment with NAC to abolish all ROS production

completely prevented induction of HO-1 by MTX but had no effect on MnSOD. An attempt to explore the effects of mitochondrial ROS using a mitochondrial ROS scavenger did not alter MnSOD or HO-1 induction but may represent a technical failure.

Therefore, the induction of cytoprotective genes in HUVEC by MTX is dependent upon an AMPK and CREB pathway. Although MTX does increase intracellular ROS production, whether this is restricted to a particular subcellular compartment or is important for the induction of MnSOD and HO-1 remains to be determined.

8.3.4 MTX increases AMPK phosphorylation and MnSOD expression *in vivo* and reduces the severity of an inflammatory vasculopathy

The experiments reported in the final chapter of this thesis explored the *in vivo* relevance of the *in vitro* findings regarding the effects of MTX on EC biology that were presented in the earlier chapters.

The male WBF1 lupus-prone mouse develops an inflammatory vasculopathy of small muscular arteries in the heart and GI tract, leading to thrombosis and organ infarction. These animals were treated with MTX 1mg/kg weekly for 4 weeks, early in the course of disease, to investigate whether MTX could reduce its severity.

MTX treatment increased vascular expression of phosphorylated AMPK α^{Thr172} and MnSOD, and reduced aortic adventitial and endothelial ICAM-1 expression. MTX treatment also attenuated organ damage and reduced the severity of the coronary vasculopathy. However, there were not enough affected animals for an effect of MTX in myocardial infarct number or size to be seen.

These results show that MTX is capable of activating AMPK in the vasculature at doses relevant to clinical practice in both *in vivo* and *in vitro* settings, and also enhancing MnSOD expression. Furthermore it can modulate the course of an inflammatory vasculopathy in the setting of a murine lupus-like illness, although whether this depends on activation of AMPK or alterations in MnSOD expression has not yet been definitively established.

8.4 Discussion

The central finding of the work presented in this thesis is the identification of a new mechanism of action of MTX that might explain why patients with RA who are treated with long-term, low dose MTX are at reduced risk of CV mortality. I have shown that MTX might exert beneficial effects on normal tissue, rather than just precipitating apoptosis in cells that we wish to get rid of. In EC MTX activates AMPK, phosphorylates CREB and induces expression of cytoprotective genes with roles in mitochondrial function and with anti-oxidant, anti-apoptotic properties. MTX did reduce EC proliferation and induced a rise in intracellular ROS levels, but protected against apoptosis induced by glucose deprivation. MTX also activated AMPK and increased MnSOD expression in the murine vasculature, and ameliorated the severity of vasculopathy in the WBF1 lupus-prone mouse.

Is MTX beneficial or harmful to the vasculature?

Although MTX has been in widespread use as a treatment for chronic inflammatory diseases for nearly 30 years, it is not completely accepted by practising physicians that MTX offers any benefit to normal tissue. Its use at high doses as a cancer chemotherapy and abortifacient drug have perpetuated a popular view that MTX is harmful, and is effective in treating inflammatory disease only because it kills rapidly dividing cells before normal cells are irreversibly damaged. Many physicians, if asked whether MTX might exert beneficial effects on normal tissue such as vascular endothelium, would think not.

There are several ways in which MTX might be harmful. First, it induces apoptosis in malignant cells, activated leucocytes, and other actively dividing cell types such as keratinocytes[380, 384]. Secondly, it increases ROS levels, and can do so to a degree that promotes apoptosis, again in activated leucocytes[380]. More pertinent to the vasculature, MTX can increase plasma homocysteine levels and interfere with the production of the eNOS co-factor BH4[388], and has anti-angiogenic properties[385]. It might therefore be reasonable to propose that MTX is likely to be harmful to the vasculature, and indeed initial studies on MTX and CVD in RA suggested CVD was increased, associating this with a rise in plasma homocysteine concentration[357].

However, clinical experience of MTX prescribed at 15-20 mg per week in rheumatological practice is altering perceptions of this drug and its long-term safety. Co-prescription of folic acid and regular monitoring of relevant blood indices has resulted in MTX being taken safely by thousands of patients over many years. Although MTX does increase homocysteine concentrations, co-prescription with folic acid reverses this[360]. Several studies have now demonstrated that the long-term low-dose MTX regimen used in RA is associated with reduced CVD[79, 361]. The use of MTX is also associated with improvement in endothelial function[363]. This evidence, combined with the long clinical experience supporting its safety, has led to the initiation of a trial of long-term low dose MTX

for the secondary prevention of CVD in patients with atherosclerosis but no underlying inflammatory condition[15].

Improvement in CV mortality with MTX may not solely be due to direct beneficial effects of the drug on endothelium. They may also reflect an anti-atherogenic action of adenosine via ligation of its A_{2A} receptor which, in addition to an anti-inflammatory action, may induce reverse cholesterol transport proteins and prevent foam cell formation[390]. MTX is also a very effective anti-inflammatory drug which successfully reduces levels of circulating cytokines and other inflammatory mediators, and the reduction in CV mortality could simply be an indirect result of its general suppression of chronic inflammation.

In the work presented in this thesis, no evidence of harm to EC was found using MTX. Although MTX did reduce cell proliferation *in vitro*, this is unlikely to be significant *in vivo* given the much lower rate of turnover of EC. MTX did not induce EC apoptosis at the doses used, and indeed protected against it when induced using glucose deprivation. Although an increase in ROS was found in cells treated with MTX, it was unclear where in the cell this occurred and whether it was physiological or not. In addition, MTX increased expression of cytoprotective genes. However, it remains to be determined whether the changes in gene expression occur because MTX promotes a more healthy internal cell milieu, or whether they are a response to a stressful effect of MTX on the cells. This is entirely possible given that MTX inhibits enzymes involved in many aspects of cell metabolism. If so, MTX may be exerting beneficial effects by inducing stress-response hormesis. This is a term borrowed from the study of longevity referring to the phenomenon whereby low-level stress, by inducing expression of protective pathways, improves resistance to subsequent challenges[89, 179].

Overall, this study supports the notion that MTX is likely to be protective to the vasculature.

Are the findings relevant to the use of MTX in clinical practice?

There are several reasons to support the idea that the effects of MTX on EC discovered in the experiments presented here may be relevant to the use of long-term low dose MTX in the treatment of chronic inflammatory disease. Firstly, phosphorylation of AMPK and changes in downstream enzymes were seen in HUVEC at MTX concentrations thought to be achievable in patient sera, and moreover increased phosphorylated AMPK and the downstream target MnSOD was observed in murine vascular tissue following MTX administration analogous to the long-term, low dose use that is so effective in chronic inflammatory disease. Secondly, the effects of MTX on MnSOD expression at least were replicated in both HUVEC and HAEC, as well as in murine vascular tissue. Thirdly, although I was not able to demonstrate an effect of MTX on pro-inflammatory gene expression *in vitro*, reduction in ICAM-1 expression was observed *in vivo*, suggesting that the MTX doses and administration used were operating as would be expected in terms of effect on inflammatory

pathways. Finally, *in vitro* AMPK phosphorylation and changes in gene expression were only observed with MTX after a 48h delay. Given that MTX, when used as an anti-inflammatory drug, has a long onset and offset of action, taking several weeks, a delayed response is consistent with an effect relevant to the use of MTX in clinical practice.

Therefore, I think it likely that the MTX-mediated AMPK activation in the vasculature that has been demonstrated is likely to be a real phenomenon in patients.

Could AMPK activation by MTX be part of its anti-inflammatory mechanism of action in addition to vascular protection?

This thesis presents data showing that for the first time that MTX activates AMPK in vascular endothelium, both *in vitro* and *in vivo*, and at concentrations relevant to clinical practice. AMPK has been associated with anti-inflammatory and desirable metabolic changes in many different systems and disease settings[261, 266]; the evidence supporting its protective role in EC was discussed in the introduction.

Recent studies have suggested that AMPK activation in cells of the immune system is associated with anti-inflammatory changes[266]. The question therefore arises whether MTX may also activate AMPK in cells of the immune system as part of its anti-inflammatory mechanism of action. This is certainly possible, particularly as the anti-inflammatory mechanism of action of MTX is still a matter of some debate. Although there is strong *in vitro* and *in vivo* evidence that adenosine accumulation is important, there is little evidence in patients of alterations in adenosine levels. In addition, in Chapter 7 AMPK phosphorylation was demonstrated in murine aortic tissue following MTX treatment. Endothelial cells are only a small proportion of the total cell content of the aorta, and so it is likely that MTX does activate AMPK in tissues other than the endothelium.

AMPK activity shifts cellular energy generation towards mitochondrial oxidative phosphorylation, which is associated with a quiescent, anti-inflammatory cellular phenotype with low energy turnover and reduced cell growth and proliferation. This is thought to be important in mediating AMPK's anti-cancer effects and is postulated to underlie its anti-inflammatory actions[266]. In particular, AMPK activation may discourage use of glycolysis and the pentose cycle for energy generation, as is seen in many activated leucocytes, and promote oxidative phosphorylation via mitochondria that generate significantly greater ATP per mole of glucose. In addition, AMPK activation may have direct anti-inflammatory actions, reducing NFκB-mediated transcription and iNOS expression in macrophages.

Is there evidence to suggest MTX might also promote some of the changes in cell phenotype that have been ascribed to AMPK activation? Certainly MTX reduces cell proliferation, although this may be due to its effects on folic acid and purine metabolism rather than through AMPK. There is also evidence that MTX, albeit at high dose, can inhibit enzymes involved in the pentose phosphate pathway[416], an alternative energy source that is used by activated macrophages and which AMPK

activation diverts cell metabolism away from. In addition, MTX and AMPK activation share similar effects on glycogen metabolism: while MTX increases glycogenolysis in rat liver, AMPK activation may promote glycogenolysis through phosphorylation and inactivation of glycogen synthase[305, 415]. Finally, MTX has broad anti-inflammatory effects in a number of settings, reducing circulating levels of cytokines and soluble cell adhesion molecules and potently inhibiting progression of chronic inflammatory diseases. Thus, MTX could mimic effects of AMPK activation in other ways as well as activating the pathway itself.

The importance of CREB, MnSOD and HO-1 in vascular protection and potential role in anti-inflammatory effects of MTX

I found that, following AMPK activation, MTX treatment increases CREB phosphorylation and binding to the MnSOD promoter, and induces MnSOD and HO-1. All three of these proteins are known to play roles in endothelial cytoprotection, and can also have anti-inflammatory effects. It is possible therefore that their induction by MTX forms part of its anti-inflammatory mechanism of action.

The importance of CREB in vascular protection was discussed in the introduction. Loss of aortic CREB is found as an early feature in rodent models of hypertension, atherosclerosis and insulin resistance[201], while cardiac-specific expression of dominant-negative CREB increases oxidative stress, mitochondrial dysfunction and mortality[202]. In the immune system, CREB is thought to exert anti-inflammatory effects by interfering with NFκB-mediated transcription[199]. CREB and the RelA component of NFκB compete for the same limited supply of co-activators, CBP and p300, and so the relative abundance of NFκB and CREB in the nucleus determines whether NFκB or CREB-mediated transcription occurs[490].

Induction of MnSOD protects against atherogenesis and ApoE^{-/-} mice deficient in MnSOD exhibit accelerated atherosclerosis[226]. Induction of MnSOD by MTX may also be involved in its anti-inflammatory effect: treating a rodent model of arthritis with MTX as well as a SOD mimetic reduced the dose of MTX needed to control disease by 10-fold[394].

Finally, a wealth of evidence, reviewed in the introduction, demonstrates the role of HO-1 in vascular protection. However, its induction is also significantly anti-inflammatory too. *Hmox1*^{-/-} mice suffer more severe versions of a variety of experimental auto-immune diseases[211], while induction of HO-1 reduces the severity of nephritis in lupus-prone mice and plays a regulatory role in RA synovial inflammation[214, 215].

Could MTX modulate mitochondrial function?

A number of the changes in EC function observed after MTX treatment suggest that MTX affects mitochondrial metabolism and possibly mitochondrial ROS production. MnSOD and UCP2, both induced in MTX-treated cells, act to control ROS production in mitochondria. The anti-apoptotic protein A1, also induced by MTX, is associated with mitochondria and controls apoptosis via the mitochondrial pathway. It is also known that AMPK signalling leads to mitochondrial biogenesis in EC, and that loss of CREB is associated with mitochondrial dysfunction[202, 310]. Finally, MTX use was associated with a rise in intracellular ROS levels, although I was not able to show whether this was localised to the mitochondria or was a more generalised phenomenon.

Mitochondria in EC are not thought to function primarily for energy generation. Instead, they signal cell responses to environmental cues, through the regulated production of ROS, for which UCP2 and MnSOD are key[124]. MnSOD controls the conversion of superoxide to H₂O₂, a relatively stable moiety that is capable of signalling. UCP2 controls the speed of electron leak by the electron transfer chain and thus the rate of ROS production[238]. Perinuclear mitochondria release ROS that can diffuse to the nucleus and alter gene expression, and H₂O₂ also plays regulatory roles in shear-stress induced vasodilation, autophagy, and inflammatory activation[124]. Therefore it is possible that MTX, by altering levels of MnSOD and UCP2, is altering mitochondrial ROS production and thus influences cell biology in other ways downstream.

AMPK activation and modulation of mitochondrial metabolism might provide a strategy for vascular protection and for reduction of inflammation in chronic inflammatory disease

A recent review of the effects of AMPK on inflammation suggested that it was through its actions on cell metabolism in the immune system that AMPK was anti-inflammatory, and proposed that modulating cell metabolism could be employed pharmacologically as an anti-inflammatory strategy in chronic inflammatory diseases[266].

The original function of the AMPK molecular ancestors is energy conservation in response to starvation. In accordance with this, AMPK activation in the immune system mimics the effects of starvation and seems to divert metabolism away from rapid glucose uptake and utilisation in glycolysis, towards mitochondrial oxidative phosphorylation and fatty acid oxidation. This change in metabolic state appears to promote a switch from a pro-inflammatory to anti-inflammatory cell phenotype. AMPK activation "tricks" the cell into behaving as if it is at risk of nutrient shortage, encouraging it to adopt a quiescent phenotype in order to survive[266].

Real starvation, or calorie restriction, is associated with benefits to the whole organism. It has long been known that calorie restriction improves longevity in *C. elegans* and in rodents. AMPK is necessary in *C. elegans* for the extension of lifespan prompted by calorie restriction. Calorie restriction

can also improve resistance to inflammatory disease: a reduction in calories by 30% in the WBF1 mouse, if started at an early age, markedly delays onset of disease and improves survival[484].

I discussed earlier the concept of stress-response hormesis: a brief or low level exposure to a stressor enhances resistance to future stressors, and at a molecular level, there is an induction by the stressor of an adaptive defensive response through alteration in gene expression[179]. Calorie restriction may be one such stressor, and there is evidence to suggest that the enhancement of lifespan secondary to calorie restriction may be mediated by stress-response hormesis. For example, rats exposed to calorie restriction have increased serum corticosterone, suggesting an organism under stress, but are resistant to carcinogenesis compared to normal rats. Mechanistically, at a cellular level, calorie restriction may operate via increasing mitochondrial ROS production, and AMPK may be involved in mediating this[126]. Schulz *et al* treated *C. elegans* with 2-deoxyglucose, reducing glucose availability and activating AMPK, increasing mitochondrial oxidative phosphorylation, mitochondrial ROS production and anti-oxidant enzyme expression, and increasing lifespan[318]. It should be noted however that glucose restriction is not experienced by mammalian cells physiologically due to hepatic gluconeogenesis and so this experimental result cannot be extrapolated directly to human tissue. However, it is possible that pharmacological activation of AMPK may be able to mimic some of these effects, in the vasculature or the immune system. If MTX does indeed exert vascular protective and anti-inflammatory effects via AMPK activation, it may be the downstream effects on cell metabolism, particularly on mitochondrial function, that are important. Indeed, altering cell metabolism via AMPK activation may represent a new strategy for vascular protection as well as reducing inflammation in the treatment of chronic inflammatory disease.

8.5 Future work

The principal novel findings presented in this thesis were of AMPK activation by MTX, leading to induction of cytoprotective genes in human EC and in the murine vasculature. To expand these findings, the following questions could be addressed:

1. Do the changes in AMPK and CREB activation and in cytoprotective gene expression demonstrated really occur in the vasculature of patients treated with MTX? This is difficult to address as it is very hard to obtain endothelial cells from patients, let alone the challenges of maintaining a MTX-induced phenotype in culture. One way to explore it however would be to study blood outgrowth endothelial cells (BOEC) from patients on MTX and those not, or if possible from the same patient before and after starting the drug.
2. The same technique of isolating and culturing blood outgrowth endothelial cells could also be used to study those from patients with AMPK disease. As these patients have a constitutively active AMPK complex, BOEC from these patients could be used as a model of AMPK over-expression to study the phenotype this produces in vascular endothelium.
3. What is the full extent of changes in gene expression in EC treated with low dose MTX? A genome-wide array could be performed to investigate this.
4. The role of CREB in the responses observed has not been fully demonstrated. In order to be certain that CREB activation is driving MnSOD expression in MTX-treated cells, a luciferase reporter assay should have been performed.
5. A number of experiments presented here suggest that one of the results of MTX treatment is alteration in mitochondrial metabolism. Is MTX altering mitochondrial ROS production and how is it doing so? What is the result of this? These questions could be addressed by using specific mitochondrial ROS scavengers to block their effects, and using mitochondrial ROS dyes to establish whether their levels are changing *in vivo*.
6. It was not directly established whether AMPK activation and MnSOD expression were required for the improvement in disease severity seen in WBF1 mice treated with MTX. This could be addressed by using an AMPK inhibitor such as Compound C concurrently. It is also interesting that calorie restriction has been reported to improve disease severity. It might be interesting to establish whether this too is because of AMPK activation.
7. Does activation of AMPK and induction of MnSOD by MTX also occur in leucocytes and does this lead to anti-inflammatory effects that might explain MTX's disease modifying action in RA and other diseases? This could be studied in a number of ways: first by treating cultured leucocytes with MTX; secondly by treating rodents with MTX and isolating and analysing their monocytes; and finally by analysing monocytes from patients on MTX.

8. If AMPK activation and alteration of mitochondrial metabolism do form a significant part of MTX's mechanism of action in both vascular protection and in limiting inflammation, why is it that alterations in metabolism have these effects and what are the underlying mechanisms? Are there other ways of altering cell metabolism by AMPK activation or mimicking its effects that could be used as a strategy for preventing atherosclerosis or treating inflammatory disease?

8.6 Conclusion

The work contained in this thesis set out to explore how best to protect patients suffering from chronic inflammatory diseases from premature CVD as a complication of their illness. I investigated the effects of VEGF on EC conditioned by high flow LSS, but did not find a consistent cytoprotective response elicited by the two together. However, I have established a novel mechanism of action of MTX in the vasculature, with activation of AMPK-CREB signalling, leading to the induction of cytoprotective genes. This may contribute to the observed benefits of MTX in reducing atherosclerotic complications in patients with RA, and move the perception of this drug from toxic to protective. MTX was originally designated an "anti-metabolite" drug because of its inhibition of folic acid metabolism. Perhaps in fact low-dose MTX is also altering cell metabolism, but to beneficial effect, via AMPK, and so this term should be re-thought.

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It is five years, nearly to the day, since I was first interviewed for a fellowship to pursue this project. In the intervening time I have gone from a lab ingenue with barely an idea of which button on a pipette did what to a relatively competent researcher who is now very keen to forge a career in science. Consequently, there are a very large number of people to thank for helping me make the transition.

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I am grateful to my parents for teaching me as a child about the satisfaction of achieving at something difficult. I do not think I would ever have considered embarking on a PhD without this mindset. I am also very thankful for my son, Hector, whose arrival rather complicated my final months in the lab, but whose presence has given me the spur to ensure I completed it. Finally, I am lucky to have the unwavering support of my husband, Costas, without whom there would probably have been no successful project and certainly no finished thesis. Thank you, Costas.

10 APPENDICES

10.1 Recipes for laboratory solutions

HBSS without glucose	8g NaCl 0.4g KCl 0.14g CaCl ₂ 0.1g MgSO ₄ ·7H ₂ O 0.1g MgCl ₂ ·6H ₂ O 0.06g Na ₂ HPO ₄ ·2H ₂ O 0.06g KH ₂ PO ₄ 0.35g NaHCO ₃ (1g d-glucose) Make up to 1l with ddH ₂ O and sterile filter
5x Krebs solution	40g NaCl 1g KCl 4.63g Na ₂ HPO ₄ ·2H ₂ O 1g KH ₂ PO ₄ Make up to 1l with ddH ₂ O
1x Krebs solution	200ml 5x Krebs 0.85ml 2.8M MgCl ₂ ·6H ₂ O (6.9g in 10ml H ₂ O) 131mg CaCl ₂ ·2H ₂ O Make up to 1l with ddH ₂ O
5x PSS	38g NaCl 6.25g NaHCO ₃ 1.38g KCl 0.82g KH ₂ PO ₄ 1.47g MgSO ₄ ·7H ₂ O 11.9g HEPES Make up to 1l with ddH ₂ O
1x PSS	200ml 5x PSS 1.88g glucose 0.23g CaCl ₂ ·2H ₂ O Make up to 1l with ddH ₂ O
Stripping Buffer	31.25ml Tris-HCl 0.5M pH6.8 50ml 10% SDS 1.77ml β-mercaptoethanol 167ml ddH ₂ O (for 250ml)

5x TAE	54g trizma base 27.5g boric acid 20ml 0.5M EDTA Make up to 1l with ddH ₂ O
10x TBS-T	10ml Tween-20 80g NaCl 24.2g trizma base Make up to 1l with ddH ₂ O; adjust to pH 7.8

10.2 Presentations and awards

The work presented in this thesis was funded by a research training fellowship awarded to me by the Wellcome Trust.

An abstract containing the major findings of this thesis won a Young Investigator Award at the British Society of Rheumatology annual conference 2014, and was presented in the "Jewels in the Crown" plenary session.

Other abstracts drawn from the results of this study won a poster prize at the Academy of Medical Sciences/ Medical Research Society/ Royal College of Physicians annual meeting for clinician scientists in training in 2012, and were presented at the biannual conference on Heme Oxygenases in Edinburgh in 2012 and at the EULAR annual congress in Paris 2014.

10.3 Word count

The word count, excluding references, is 57 650.

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