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Investigation of the cAMP-mediated Inhibitory Mechanism on the Signalling Pathways of 2 Cytokines; IL-6 and Leptin in Endothelial Cells

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Abstract

There is a wealth of evidence to support the anti-inflammatory properties of the prototypical second messenger cyclic-AMP (cAMP), notably with regard to endothelial function. Many studies have shown that cAMP can limit vascular permeability by enhancing barrier function and reducing pro-inflammatory effects of cytokines. Although the protective effects of cAMP elevation on limiting endothelial dysfunction have been well documented, the exact molecular mechanisms remain unclear.

Using two endothelial cell types, namely human umbilical vein endothelial cells (HUVECs) and a novel human endothelial angiosarcoma-derived cell line (AS-M), this study has further characterised the cAMP-mediated inhibitory mechanism on the signalling pathways of two cytokines; interleukin-6 (IL-6) and leptin. Both cytokines have been implicated in the regulation of the immune response and both have been shown to play important pathological roles in various inflammatory diseases.

In preliminary studies, cAMP elevation was shown to induce suppressor of cytokine signalling 3 (SOCS3) in HUVECs. Further investigation of this SOCS protein in the context of IL-6 and leptin signalling in endothelial cells would be of interest in terms of possibly elucidating the molecular mechanisms underlying the protective effects of cAMP. Results from this study demonstrated a cAMP-mediated inhibition of soluble IL-6Rα (sIL-6Rα)/IL-6-stimulated extracellular regulated mitogen-activated protein kinase 1, 2 (ERK1,2) and signal transducer and activator of transcription 3 (STAT3) activation in HUVECs, which was independent of cAMP-dependent protein kinase A (PKA). Instead, results demonstrated the involvement of the other major cAMP sensor; exchange protein activated by cAMP 1 (Epac1). Moreover, this inhibition was shown to be SOCS3-dependent. There also appeared to be a requirement for ERK1,2 activation in the cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated STAT3 activation in HUVECs. In contrast to these findings, cAMP-mediated inhibition of leptin-stimulated STAT3 activation in HUVECs was shown to occur *via* a SOCS3-independent mechanism. The responses to cAMP elevation on sIL-6Rα/IL-6- and leptin-stimulated ERK1,2 activation in AS-Ms were variable, since basal levels of ERK1,2 activation were high. Furthermore, the responses to cAMP elevation on

sIL-6Rα/IL-6- and leptin-stimulated STAT3 activation in AS-Ms were either very modest or showed no effect, respectively. SOCS3 was not shown to be involved in the cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation in AS-Ms.

In conclusion, this study further characterised the cAMP-mediated inhibitory mechanism in HUVECs and AS-Ms, with a particular focus on the ERK1,2 signalling pathway of IL-6 and leptin. Despite varying results between both cell types, this study also identified AS-Ms as a useful and tractable cell model to study in the context of endothelial biology. Thus, a potentially new pathway has been identified which inhibits cytokine receptor activation of ERK1,2 and STAT3 in endothelial cells. A better understanding of this mechanism could contribute towards new therapeutics in the area of chronic inflammatory diseases, such as atheroscleriosis.

I would like to dedicate this thesis to my two grandads; Brian Miles and Elwyn Woolson who passed away of heart disease.

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Abbreviations

AAA	Abdominal aortic aneurism
AC	Adenylyl cyclase
AS-M	Angiosarcoma-derived cell line
ATF1	Activating Transcription Factor 1
ATP	Adenosine 5’ triphosphate
BCA	Bicinchoninic acid
BMI	Body mass index
BPAECs	Bovine pulmonary artery endothelial cells
BSA	Bovine serum albumin
CAD	Coronary atherosclerosis
cAMP	Cyclic 3’, 5’ adenosine monophosphate
C/EBP	CCAAT/enhancer-binding protein
CHD_1	Coronary heart disease
CHD_2	Cytokine homology domain
CHO	Chinese hamster ovary
CIS	Cytokine-inducible SH2 domain-containing protein
CME	Clathrin-mediated endocytosis
CNS	Central nervous system
cPLA2	Cytosolic phospholipase A2
CREB	cAMP response element binding protein
CVD	Cardiovascular disease
CVR	Coronary vascular resistance
DB	Diabetes
DNA	Deoxyribonucleic acid
DUSP	Dual specificity phosphatase
DVT	Deep vein thrombosis
EC	Endothelial cell
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
EGM	Endothelial growth medium
EGTA	Ethyleneglycol-bis(2-aminoethyl)-N,N,N’,N-tetra acetic acid

EIG	Early immediate gene
eNOS	Endothelial nitric oxide synthase
Epac	Exchange protein activated by cAMP
EPO	Erythropoietin
ERK	Extracellular signal-regulated protein kinase
ET-1	Endothelin-1
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
F-III	Fibronectin type III
FRET	Fluorescence resonance energy transfer
GAP	GTPase-activating protein
G-CSF	Granulocyte-colony stimulating factor
GDP	Guanine 5’ diphosphate
GEF	Guanine nucleotide exchange factor
GHR	Growth hormone receptor
GPCR	G protein-coupled receptor
Grb2	Growth receptor bound 2
GTP	Guanosine 5’ triphosphate
H89	N-[2-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide
HAECs	Human aortic endothelial cells
HLMECs	Human lung microvascular endothelial cells
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
INF	Interferon
IRS1/2	Insulin receptor substrate 1/2
JAK	Janus Kinase
kDa	Kilo Dalton
KIR	Kinase Inhibitory region
KSR	Kinase suppressor of Ras
LIF	Leukaemia-inhibitory factor
LPS	Lipopolysaccharide
LR	Leptin receptor

MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEFs	Murine embryonic fibroblasts
MEK	Mitogen-activated protein kinase kinase
MKP-1	Protein kinase phosphatase 1
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NS	Noonan syndrome
ORL1	Opioid receptor-like 1
PAD	Peripheral arterial disease
PAI-1	Plasminogen activator inhibitor-1
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PEA-15	Phosphoprotein enriched in astrocytes 15kDa
PGE1	Prostaglandin E1
PIAS	Protein inhibitors and activators of STATs
PI3K	Phosphatidylinositol-3-kinase
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myrisate 13-acetate
PRLR	Prolactin receptor
RA	Rheumatoid arthritis
RCE	Raft/caveolae-mediated endocytosis
RIPA	Radioimmunoprecipitation buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSK2	p90 ribosomal S6 kinase 2
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B

Sef	Similar expression to fgf genes
SH2	Src-homology 2
siRNA	Small interfering RNA
SMC	Smooth muscle cell
SOCS	Suppressor of cytokine signalling
Sos	Son of sevenless
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
TBS	TRIS-buffered solution
TCF	Ternary complex factor
TEMED	N, N, N', N' –tetramethylethylenediamine
TF	Tissue factor
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TOA	Thromboangiitis obliterans
TRIS	Hydroxymethyl-aminomethane
VEGF	Vascular endothelial growth factor
WT	Wildtype

Standard one letter and three letter amino acid codes have been used throughout.

Chapter 1

Introduction

1.1 Endothelium Function

The endothelium is the largest organ in the body, lining the entire vascular system, from the heart to the smallest capillary. It forms the crucial barrier between the bloodstream and underlying tissues, ultimately controlling the passage of materials into and out of the vessel.

The endothelium actively regulates vascular tone, the homeostasis of which is maintained by the release of vasoconstrictors, such as endothelin, prostaglandins and reactive oxygen species (ROS), and vasodilators such as nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (Mombouli & Vanhoutte, 1999). In its healthy state, the endothelium maintains vascular tone and function by controlling the balance between vasodilation and vasoconstriction, anti-thrombosis and pro-thrombosis, anti-inflammation and pro-inflammation, cell growth inhibition and cell growth promotion, and also anti-oxidation and pro-oxidation. The endothelium achieves this by expressing constitutive or induced molecules on its surface, such as adhesion molecules, and secreting soluble mediators such as vasodilators and vasoconstrictors, or cytokines and growth factors. One of the most important vasoactive substances is NO, which is synthesised by the endothelial NO synthase (eNOS). NO is not only a crucial mediator of endothelium-dependent vasodilation, described initially as endothelium-derived relaxing factor (EDRF) (Palmer *et al.*, 1987), but also has important roles in inflammation and thrombosis. These include inhibition of leukocyte and platelet adhesion to the endothelium, inhibition of platelet aggregation (Alheid *et al.*, 1987; Kubes *et al.*, 1991; Radomski *et al.*, 1987), and suppression of the expression of plasminogen activator inhibitor-1 (PAI-1), a prothrombotic protein in smooth muscle cells (SMC) (Bouchie *et al.*, 1998). Impaired NO bioavailability is believed to play a major part in endothelial dysfunction (Davignon & Ganz, 2004). Endothelial dysfunction is characterised by impaired vasodilation and increased permeability, leukocyte adhesion and cytokine release. It represents an imbalance in the actions of vasoconstrictors versus the actions of vasodilators. Assessment of endothelial dysfunction is primarily based on changes in vasomotion, which is also representative of all other impaired functions of the endothelium (Landmesser *et al.*, 2004). The resulting dysfunction is associated with many conditions, such as hypertension (Lockette *et al.*, 1986; Luscher, 1990), elevated levels of low density lipoprotein and oxidised

lipoproteins (Gilligan *et al.*, 1994), elevated levels of homocysteine (McDowell & Lang, 2000), insulin resistance and type 2 diabetes (Jansson, 2007), smoking (Hutchison, 1998) and obesity (Sivitz *et al.*, 2007).

As mentioned previously, decreased NO bioavailability may be considered a crucial mechanism of endothelial dysfunction. Reactive oxygen species (ROS), so-called oxidative stress contributes greatly to endothelial dysfunction through the sequestration of NO. Thus, when the overall balance between oxidative stress and the anti-oxidative defense mechanism is upset, NO bioavailability is reduced and endothelium function is impaired. Mechanisms of oxidative stress leading to decreased NO bioavailability are discussed further in Chapter 1.1.3.

Endothelial dysfunction is believed to underpin the development of various inflammatory diseases including sepsis (Aird, 2003), diabetic retinopathy (Hsueh & Anderson, 1992) and atherosclerosis, which is discussed hereafter in the context of vascular disease.

1.1.1 Vascular Disease

Vascular disease encompasses a variety of diseases including abdominal aortic aneurism (AAA), deep vein thrombosis (DVT), thromboangiitis obliterans (TAO) and peripheral arterial disease (PAD). However, it is the PAD atherosclerosis that represents the single, most important cause of coronary heart disease (CHD_1) and stroke to date. CHD_1 and stroke are the two main forms of cardiovascular disease (CVD). CVD is the major cause of death in the UK and accounts for over 208,000 deaths each year. 48% of all deaths from CVD are caused by CHD_1 and 28% from stroke. Despite a 30 year decline, the death rate for CHD_1 in the UK is still one of the highest in Western Europe and costs the health care system millions of pounds each year (in 2003 costs accrued to around £3,500 million; www.heartstats.org).

Atherosclerosis is a chronic inflammatory disease of the arteries and, depending on which arteries are affected, results in different clinical manifestations. For example, atherosclerosis in the coronary arteries can cause angina pectoris and myocardial infarction, while atherosclerosis in the cerebrovascular circulation may lead to a transient ischaemic episode or stroke.

1.1.2 Endothelial Dysfunction and Atherosclerosis

There is an abundance of evidence to suggest that atherosclerosis is strongly linked to endothelial dysfunction. The study by Ludmer *et al*. (1986) was one of the first to show this correlation in humans. This showed that whereas there was a dose-dependent dilation of coronary arteries treated with acetylcholine in patients without coronary disease, a paradoxical *vasoconstriction* was observed in patients with mild to advanced coronary atherosclerosis, indicative of an impaired endothelium-dependent vasomotion (Ludmer *et al.*, 1986). It has also been demonstrated that many of the risk factors that predispose to atherosclerosis have the potential to cause endothelial dysfunction (e.g. hypertension, hypercholesterolemia, diabetes mellitus, smoking and family history) (Davignon & Ganz, 2004; Vita *et al.*, 1990). Thus it is not surprising that endothelial dysfunction has been shown to be associated with clinical events of atherosclerosis and may actually predict the risk of cardiac events. Several studies have shown that acute clinical outcomes of atherosclersosis, such as myocardial infarction and cardiovascular death can be predicted by endothelial dysfunction (Halcox *et al.*, 2002; Suwaidi *et al.*, 2000). In these studies, intracoronary acetylcholine was administered to patients with mildly diseased coronary arteries or patients undergoing cardiac catheterisation for investigation of chest pain, and a number of parameters, such as intravascular ultrasound examination, coronary blood flow and coronary angiography were assessed to determine endothelial vasodilation. The results demonstrated that coronary endothelial dysfunction independently predicts acute cardiovascular events. Furthermore, improvement of endothelial vasodilation in patients surviving an acute coronary syndrome was associated with significantly less cardiac events (Fichtlscherer *et al.*, 2004). These studies support the idea that endothelial dysfunction plays a crucial role in the progression of atherosclerosis. Furthermore, the findings that endothelial dysfunction in patients with coronary risk factors precedes angiographic or ultrasonic evidence of atherosclerostic plaque also suggests that it constitutes an early event in disease pathogenesis (Celermajer *et al.*, 1994; Reddy *et al.*, 1994).

1.1.3 The Endothelium and Inflammation

As mentioned previously, endothelial dysfunction is manifest as impaired endothelium-dependent vasomotion and reflects an impairment of important

endothelial functions. Of particular relevance to the present study is the endothelium's anti-inflammatory role. In its normal, healthy state the endothelium actively regulates vascular inflammation. However, upon damage the endothelium assumes a pro-inflammatory phenotype. In this state, the endothelial cells become activated and start to exhibit oxidative stress and an increased adhesiveness to circulating leukocytes.

Oxidative stress, in terms of superoxide (O_2^-) production, increases greatly in activated endothelial cells. This can be caused by a number of factors, such as uncoupling of eNOS, whereby O_2^- is generated, instead of NO. Thus, O_2 reduction by eNOS is uncoupled from NO formation, and a functional eNOS is converted into a dysfunctional O_2^--generating enzyme that contributes to vascular oxidative stress. Uncoupling has been associated with the reduced availability of the eNOS co-factor BH4 (Schmidt & Alp, 2007). The resultant O_2^- can react with NO to produce peroxynitrite ($ONOO^-$), a potent oxidant, which in turn leads to uncoupling and enzyme dysfunction, possibly *via* oxidation of tetrahydrobiopterin (BH4), which reduces its bioavailability (Beckman *et al.*, 1990; Laursen *et al.*, 2001; Rubanyi & Vanhoutte, 1986; Schmidt & Alp, 2007). Thus, a vicious cycle is established whereby the vasoprotective effects of NO are precluded (Kubes *et al.*, 1991). In support of this, many studies have demonstrated that BH4 can restore endothelial dysfunction in patients with hypercholesterolemia, diabetes or coronary heart disease (Heitzer *et al.*, 2000; Maier *et al.*, 2000; Stroes *et al.*, 1997), implicating impaired eNOS function (resulting from decreased availability of BH4) in endothelial dysfunction (Schmidt & Alp, 2007). Furthermore, levels of an endothelial-derived substance, endothelin-1, are increased in response to oxidative stress (Kahler *et al.*, 2000). In addition to mediating vasoconstriction, endothelin-1 has been shown to activate polymorphonuclear secretion and adhesion and macrophage activity (Zouki *et al.*, 1999). Thus, oxidative stress greatly contributes towards endothelial dysfunction and this is supported by the findings that anti-oxidant treatment in the form of ascorbic acid can rescue endothelial-dependent vasodilation in patients with hypercholesterolaemia or coronary heart disease (Levine *et al.*, 1996; Ting *et al.*, 1997).

The attachment of leukocytes to the endothelium is an essential process for the propagation of a normal inflammatory response in response to infection or injury. However when this response is exaggerated or inefficiently terminated, it can lead

to the development of a chronic inflammatory state. Leukocyte interaction with the endothelium involves a series of highly coordinated adhesive and signalling events mediated by specific groups of adhesion molecules, including selectins, integrins and cellular adhesion molecules (CAMs). Intracelluar adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and E-selectin are all upregulated by endothelial cells in response to pro-inflammatory cytokines, such as IL-1β and TNFα, to promote leukocyte (monocytes and neutrophils) adhesion and transmigration (Cotran & Pober, 1990; Hakkert *et al.*, 1991; Luscinskas *et al.*, 1991; Takahashi *et al.*, 1994). Moreover, IL-1β and TNFα stimulate the production of secondary cytokines, including IL-6 which is involved in activating the acute phase response (detailed in section 1.3.2) (Jarvisalo *et al.*, 2006). The chemokine "monocyte chemoattractant protein-1" (MCP-1/CCL2) is also upregulated by IL-1β and TNFα in endothelial cells (Rollins *et al.*, 1990). MCP-1 not only stimulates the recruitment of monocytes, but has also been shown to increase endothelial cell proliferation (Weber *et al.*, 1999). Additionally, T cells are also recruited to the activated endothelium, where they secrete pro-inflammatory cytokines such as IL-1β, TNFα and also IFNγ (Tesfamariam & DeFelice, 2007). Although described very briefly, all of these inflammatory processes greatly contribute to a chronic and persistent state of inflammation that manifests itself in disease, notably atherosclerosis.

1.2 Adenosine-3',5'-cyclic monophosphate (cyclic AMP)

Cyclic AMP (cAMP) is one of the oldest signalling molecules known. It was first discovered as an intracellular mediator of hormone action in 1957 (Sutherland & Rall, 1958). Since then, numerous studies have shown that cAMP acts as a second messenger for a plethora of hormones, neurotransmitters and growth factors to regulate a vast array of cellular processes, including proliferation, differentiation, secretion, apoptosis, adhesion and migration (Beavo & Brunton, 2002). cAMP can be synthesised *via* G protein coupled receptor (GPCR) activation of adenylyl cyclases (AC), of which there are 9 membrane-bound isoforms (Sunahara & Taussig, 2002) and 1 soluble form, which is insensitive to regulation by G-protein and forskolin (a potent activator of AC, detailed in Chapter 3) (Buck *et al.*, 1999). Each AC molecule comprises 12 transmembrane sections

and 2 cytosolic domains (C1 and C2), with C1 and C2 forming the catalytic core (Cooper, 2003) (Figure 1.1). AC catalyses the conversion of adenosine 5' triphosphate (ATP) to cAMP by formation of an intramolecular 3'–5' phosphodiester bond, whereas enzymes known as cyclic nucleotide phosphodiesterases (PDEs) hydrolyse cAMP to adenosine 5'–monophosphate (5'-AMP). Phosphodiesterases thereby act to degrade cAMP *via* hydrolysis of the 3' cyclic phosphate bond. PDEs may be considered a diverse set of enzymes, comprising 11 families of which there are 21 gene products. Alternative transcriptional start sites and alternative splice variants are believed to give rise to many more than 21 mRNA transcripts or protein products (Bender & Beavo, 2006). The 11 families of PDEs can be divided into 3 groups, based on their substrate specificity; (1) cAMP-specific PDEs including PDE4, PDE7 and PDE8 (2) cGMP-specific PDEs including PDE5, PDE6 and PDE9 and finally (3) dual specificity PDEs including PDE1, PDE2, PDE3, PDE10 and PDE11 (Bender & Beavo, 2006). Thus cAMP levels are tightly regulated by the co-ordinated actions of both ACs and PDEs in the cell.

cAMP exerts its numerous biological effects by binding and activating what was initially believed to be the only cAMP effector, cAMP dependent protein kinase A (PKA) (Walsh *et al.*, 1968). However, other targets of cAMP have since been identified, namely "exchange proteins directly activated by cAMP" (Epacs) (de Rooij *et al.*, 1998), cAMP-regulated ion channels (Fesenko *et al.*, 1985) and the Ras or Rap guanine nucleotide exchange factor, cyclic nucleotide rasGEF (CNrasGEF) (Pham *et al.*, 2000). The best characterised cAMP sensors, namely PKA and Epac will be discussed in more detail further on in this Chapter. For such a limited number of cAMP effectors, there is an extensive number of cellular responses. These pleiotropic effects of cAMP pose the question of how specificity is achieved. Interestingly, there is increasing evidence to support the notion of cAMP compartmentalisation within the cell. A particular focus of this work has been the identification of macromolecule complexes within defined regions of the cell, which comprise proteins of the cAMP signalling pathway. These spatially confined signalling complexes may only permit the selective activation of certain signaling pathways (discussed further in Section 1.2.2). However, this is only a partial explanation of how cAMP specificity may be achieved. Another consideration is the freely diffusible nature of cAMP in the cell. The emergence of

Fluorescence resonance energy transfer (FRET) approaches has enabled the identification of discrete pools of cAMP in the cell (Zaccolo *et al.*, 2006). The restricted diffusion of cAMP to these pools is believed to be largely due to the actions of PDEs. PDEs have been shown to be present within multiprotein signaling complexes in the cell, acting to quench cAMP and controlling the intracellular gradients of cAMP in the cell.

cAMP has long been known to act as an endogenous 'off' signal of the inflammatory response, preventing the effects of chronic inflammation (Moore & Willoughby, 1995) and a large body of research supports this notion. Some examples of the inhibitory effects of cAMP elevation include the suppression of lysosomal enzymes, ROS and platelet-activating factor (PAF) from neutrophils (Fonteh *et al.*, 1993; Nielson, 1987; Weissmann *et al.*, 1971), the reduction of cytokines and nitric oxide released from macrophages (Bulut *et al.*, 1993; Renz *et al.*, 1988), the inhibition of eosinophil respiratory burst activity (Dent *et al.*, 1991) and the inhibition of cytotoxic T lymphocyte activation (Wisloff & Christoffersen, 1977). Of relevance to the present study is the role that cAMP plays in modulating inflammation in the context of endothelial cell function. Studies have shown that cAMP can limit vascular permeability by enhancing barrier function, and reduce pro-inflammatory effects of cytokines (Blease *et al.*, 1998; Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Morandini *et al.*, 1996; Pober *et al.*, 1993). For example, TNFα-stimulated expression of E-selectin and VCAM-1 has been shown to be inhibited by cAMP elevation in human lung microvascular endothelial cells (HLMECs) and HUVECs (Blease *et al.*, 1998; Morandini *et al.*, 1996; Pober *et al.*, 1993). Additionally, inhibition of neutrophil adherence to TNFα-stimulated HLMECs in response to cAMP elevation has been observed (Blease *et al.*, 1998).

1.2.1 PKA

PKA is a ubiquitous cellular kinase that phosphorylates serine and threonine residues in response to cAMP. It is the most well studied cAMP effector and was initially described during a number of studies in the 1950s and '60s carried out by Edwin G. Krebs *et al*. looking at the glycogenolytic enzyme glycogen phosphorylase and how it was regulated in skeletal muscle (Krebs *et al.*, 1959; Krebs *et al.*, 1966). These experiments suggested the involvement of a cAMP-

induced kinase enzyme, but it was not until later experiments conducted by Walsh *et al*. (1968) and Reimann *et al.* (1971) that this cAMP-induced kinase enzyme was identified as cAMP-dependent protein kinase (Reimann *et al.*, 1971; Walsh *et al.*, 1968), now called PKA.

1.2.2 PKA Structure and Regulation

PKA comprises 2 catalytic (C) subunits and 2 regulatory subunits (R), which form a heterotetramer (Taylor *et al.*, 1990). In mammals, 3 genes for the catalytic subunit exist (Cα, Cβ and Cγ) and 4 genes for the regulatory subunit (RIα, RIβ, RIIα and RIIβ). cAMP binds to two binding sites on each R subunit, which relieves the contact with the C subunits, causing the release of the active C subunits. Thus, unstimulated PKA is held in an inactive state *via* conformational constraints imposed by the R subunits, until cAMP binds and liberates the C subunits (Granot *et al.*, 1980). The free catalytic subunits are then able to phosphorylate serine and threonine residues in adjacent PKA substrates, for example C-Raf/Raf1, which is phosphorylated at specific serine residues (Dhillon *et al.*, 2002b). Thus, until cAMP is elevated in the cell, the regulatory subunits inhibit PKA activity. In addition to this, the regulatory subunits also bind to scaffolding proteins known as A-Kinase Anchoring Proteins (AKAPs), which anchor the PKA holoenzyme to specific subcellular structures (Colledge & Scott, 1999; Michel & Scott, 2002). This AKAP-regulatory subunit interaction controls intracellular localisation of PKA and thus regulates PKA-mediated biological effects. For example, the muscle-specific A-kinase anchoring protein (mAKAP) has been proposed to anchor PKA to a cellular localisation, wherein PDE4D3 serves as an adapter protein for Epac1 and ERK5, which together generates a cAMP-responsive signalling complex, present in cardiomyocytes. This complex integrates 2 cAMP-mediated pathways, whereby PKA and Epac can become activated *via* ERK5 phosphorylation of PDE4D3, which suppresses phosphodiesterase activity leading to increased cAMP. Activated PKA can then phosphorylate PDE4D3, which increases its affinity for cAMP and decreases localised cAMP levels and activated Epac1 can inhibit ERK5, thus preventing continued inactivation of PDE4D3. Hence, a feedback loop is generated, which causes cAMP levels to fall when they become elevated. (Dodge-Kafka *et al.*, 2005). This study partly contributes towards the

understanding of the present thesis' results and will be discussed further in the Results chapters.

Further to these regulatory effects of the PKA regulatory subunits, another level of regulation can be exerted *via* endogenous PKA inhibitors termed "protein kinase inhibitor" (PKI) peptides. Walsh and his colleagues discovered PKI soon after the discovery of PKA in 1971 (Walsh *et al.*, 1971). There are 3 isoforms termed PKIα, PKIβ and PKIγ (Beale *et al.*, 1977; Collins & Uhler, 1997; Olsen & Uhler, 1991), all of which act to inhibit the phosphorylation of PKA substrates by competitively binding to the substrate binding site of the free C subunit following dissociation from the R subunits. Thus, PKI inhibition only occurs in the presence of cAMP, after the regulatory and catalytic subunits have been separated (Ashby & Walsh, 1972; Ashby & Walsh, 1973). As well as acting as a potent competitive inhibitor of PKA, PKI has also been shown to mediate the export of free catalytic subunits of PKA from the nucleus to the cytoplasm (Fantozzi *et al.*, 1994), wherein these catalytic subunits can reassociate with the regulatory subunits to re-form the PKA holoenzyme and thus restore cAMP regulation (Dalton & Dewey, 2006). This is possible because PKI has a nuclear export signal, which causes the ATP-dependent export of the PKI-bound catalytic subunit out of the nucleus and into the cytoplasm (Wen *et al.*, 1994). Of interest, endothelial cells from pulmonary artery, foreskin microvascular and brain microvascular sources have all been shown to express the PKIα and PKIγ isoforms (Lum *et al.*, 2002). Furthermore, overexpression of PKI in human microvascular endothelial cells has been shown to abolish cAMP-mediated inhibition of endothelial permeability, implicating PKA in regulating endothelial barrier function (Lum *et al.*, 1999).

1.2.3 Epac

Epac was discovered in 1998 by 2 independent groups, as a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Rap1 belongs to the Ras superfamily of small GTPases, comprising more than 150 members. This family is sub-divided into 5 main groups, based on sequence and functional similarities; (1) Ras (2) Rho (3) Rab (4) Arf and (5) Ran. The Ras sub-family consists of 36 members, including R-Ras, Ral and Rap proteins (Wennerberg *et al.*, 2005). All members of the Ras superfamily function as molecular switches, cycling between inactive GDP-bound forms and

active GTP-bound forms. This cycle is regulated by guanine nucleotide exchange factors (GEFs) and a combination of GTPase activating proteins (GAPs) and intrinsic GTPase activity. GEFs dissociate the bound GDP to allow the association of the more abundant GTP, thus activating the G protein, whereas the intrinsic GTPase activity, together with GAPs catalyse GTP hydrolysis and subsequently inactivate the protein (Boriack-Sjodin *et al.*, 1998). The GTPase Rap1 was first identified as an antagonist of Ras, whereby overexpression of Rap1 was shown to suppress the transformation of cells with an oncogenic K-Ras mutation (Kitayama *et al.*, 1989). Since then, Rap1 and its regulation by Epac has been the focus of much research. More than 30 GEFs capable of activating Ras members have been identified and many of these GEFs activate Rap (Quilliam *et al.*, 2002). Epac1 and Epac2 represent a novel class of GEFs, since these are the only Rap1 GEFs activated directly by cAMP. This was an important discovery in terms of helping to explain PKA-independent effects of cAMP documented in numerous studies (Gonzalez-Robayna *et al.*, 2000; Kashima *et al.*, 2001; Laroche-Joubert *et al.*, 2002; Rangarajan *et al.*, 2003; Schmidt *et al.*, 2001).

1.2.4 Epac Structure and Regulation

Epac 1 and 2 are structurally very similar, with each protein containing; (1) a cAMP-binding domain, which is similar to that found in the regulatory domain of PKA, so-called B-site (2) a CDC25 homology domain, which exhibits GEF activity for Rap proteins, (3) a Ras association (RA) domain, which facilitates binding to Ras, (4) a Ras exchanger motif (REM), which is involved in interaction with the GEF domain and finally (6) a Disheveled/Egl-10/pleckstrin (DEP) domain, which is required for membrane association (Bos, 2006) (Figure 1.2). However, differences between both proteins do exist; for example, although both Epacs have been shown to possess a putative RA domain, only Epac2 has been shown to bind H-, K- and N-RasGTP *via* this domain (Li *et al.*, 2006). This interaction has been shown to promote the translocation of Epac2 from the cytosol to the plasma membrane, causing the subsequent activation of a pool of membrane-localised Rap1 (Li *et al.*, 2006). Thus, it appears that only Epac2 has a functional RA domain (Epac1 lacks key residues involved in Ras association; (Wohlgemuth *et al.*, 2005). Furthermore, Epac2 differs from Epac1 by having an additional cAMP binding domain, the so-called A-site, which has a lower affinity for cAMP (87μM)

than the B-site cAMP-binding domains of Epac1 and Epac2 (4 and 1.2 μM, respectively) (de Rooij *et al*., 2000). The function of this second cAMP binding domain has yet to be determined (Rehmann *et al*., 2003a). Additionally, the DEP domain of Epac1 has been shown to target Epac1 to the plasma membrane (de Rooij *et al*., 2000), whereas the function of the DEP domain of Epac2 appears to be less clear, since membrane association of Epac2 has only been observed when Epac2 is over-expressed (Li *et al*., 2006). In keeping with localization differences between both Epac1 and Epac2, Epac1 has also been shown to possess a mitochondrial localisation signal at its N terminus (Qiao *et al*., 2002) which is absent from Epac2. This may explain the lack of punctuate staining of Epac2 in cells (Li *et al*., 2006). The localization of Epac1 appears to be dependent on the cell cycle, showing membrane and mitochondria localization during interphase in COS-7 cells (Qiao *et al*., 2002).

Despite their differences, both Epac1 and Epac2 are capable of activating Rap1 and Rap2 in response to cAMP (de Rooij *et al*., 2000). Binding of Rap to Epac is believed to occur following a conformational change induced by cAMP binding, which relieves the auto-inhibition imposed by the N-terminal regulatory region. This has been proposed based on the crystal structure of Epac2 in the absence of cAMP (Rehmann *et al*., 2006). Thus, the closed structure of Epac2 displays a covering of the regulatory region over the predicted binding site, which appears to sterically hinder access of Rap. cAMP binding is believed to disrupt this inhibition, although the structure of Epac in the presence of cAMP has yet to be determined.

1.2.5 Roles of Epac in Endothelial Biology

Of relevance to endothelial biology, endothelial cells only appear to express Epac1 (Fang & Olah, 2007). Studies in this area have revealed Epac1 to be important in the modulation of endothelial barrier function. Rap1 has been shown to have many downstream effectors, including scaffolding proteins, such as AF6 (Boettner *et al.*, 2003), RapL (Katagiri *et al*., 2003) and Riam (Lafuente *et al*., 2004). These scaffolding proteins have been implicated in cell adhesion and cell-cell junction formation and hence, a role for Epac in these processes has been thoroughly investigated. One of the first studies to demonstrate a role for Epac in cell adhesion was conducted by Rangarajan *et al*. (2003) showing that integrin-mediated cell adhesion was enhanced *via* an Epac-Rap1 pathway following cAMP

elevation (Rangarajan *et al*., 2003). However, this study was carried out in an ovarian carcinoma cell line. To assess the role of Epac in endothelial biology, various studies have been conducted in endothelial cells, wherein endothelial barrier function is crucial for the maintenance of a healthy endothelium. A defining feature of endothelial dysfunction is increased vascular permeability, leading to inflammatory cell infiltration and chronic inflammation. Several studies have demonstrated an Epac1-mediated enhancement of endothelial barrier function. More specifically, Epac1-Rap1 mediated increases in vascular endothelial (VE) cadherin-dependent cell adhesion and cortical actin have been observed (Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005). Structurally, the endothelial cell barrier is made up of adherens junctions (AJ) and tight junctions. VE-cadherin constitutes the AJ and mediates calcium-dependent intercellular adhesions, which is strengthened by the association of VE-cadherin with the actin cytoskeleton. Thus, Epac1 has been implicated in this process, demonstrating for the first time a PKA-independent signalling pathway in endothelial cells, which regulates vascular permeability. Although Rap1 has been implicated in this pathway, it has also been reported that Epac1 can bind and activate R-Ras, which has also been implicated in integrin-mediated cell adhesion (Lopez De Jesus *et al*., 2006).

1.3 Cytokines

Cytokines are small soluble regulatory proteins that function as intercellular messengers, predominantly in the immune system. The vast majority of cytokines are referred to as “interleukins”, a name that traditionally implied that they were secreted by and acted upon leukocytes. Chemokines are another group of cytokines, defined by the role they play in chemotaxis. However, cytokines can be secreted by numerous cell types and exert a plethora of physiological effects, such as the development of cellular and humoral immune responses, induction of the immune response, control of cellular proliferation and regulation of haematopoiesis.

Classification of cytokines can be based on a number of parameters, including, structural differences (Heinrich *et al*., 1998). The “four α-helix bundle” family comprise cytokines with a four α-helix bundle structure consisting of 2 pairs of anti-parallel α-helices. These cytokines can be further sub-divided into short chain, long chain and eight α-helices groups (Bravo & Heath, 2000). The short chain

group comprise cytokines with α-helices of 8-10 residues in length, such as IL-2, IL-3 and IL-4, whilst the long chain group comprise cytokines with α-helices of 10-20 residues in length, such as growth hormone (GH), erythropoietin (EPO), granulocyte colony-stimulating factor (GCSF) and the gp130 cytokines. The third group include cytokines, such as IL-5 and interferon-γ, which have a duplication of the 4-helix bundle and thus, a total of 8 α-helices.

Cytokine receptors can again be classified according to structure and on this basis may belong to any one of five receptor families; (1) Immunoglobulin superfamily, (2) Class I family, (3) Class II family, (4) TNF superfamily and lastly (5) Chemokine receptor family. The class I receptor family comprise most of the receptors that function in the immune and haematopoetic systems and possess a characteristic cytokine receptor homology domain (CHD_2), which is situated in the extracellular domain. The CHD_2 comprises 7 β-strands that are arranged as anti-parallel β-sandwiches, connected by a proline-rich sequence. The defining feature of this structure is the WSXWS box, (Trp-Ser-Xaa-Trp-Ser, where X is a nonconserved residue), present in the C-terminal end and a conservation of cysteine residues (Bazan, 1990).

1.3.1 Interleukin-6 (IL-6)

IL-6 belongs to the IL-6 family of haematopoietic cytokines, a sub-family of the four α-helix bundle cytokines; the long chain group, comprising IL-6, IL-11, oncostatin M (OSM), leukaemia inhibitory factor (LIF), cardiotrophin 1 (CT-1), ciliary neurotrophic factor (CNTF), and cardiotrophin-like cytokine (CLC). IL-6 has a wide variety of biological effects and this is in part reflected in its nomenclature. For example, IL-6 was cloned in 1986 (Hirano *et al*., 1986) and called B cell stimulatory factor-2 (BSF-2), because of the crucial role it played in the differentiation of B cells to immunoglobulin-producing cells (Muraguchi *et al*., 1981). However, other groups cloned the same protein under the name of interferon β2 protein (IFNβ2) because it was shown to induce interferon (IFN) activity (Zilberstein *et al*., 1986). Later, hybridoma growth factor (HGF) (Van Damme *et al*., 1987) was found to be the same protein, so-called because this group found that it induced myeloma and plasmacytoma growth, and also induced

acute phase reactants in hepatocytes. When these proteins were discovered to be identical the names were unified as IL-6 (Naka *et al*., 2002).

In general, the IL-6 family of cytokines have been demonstrated to play important roles in haematopoiesis, embryonic development, fertility, liver and neuronal regeneration and, of particular relevance to the present study, inflammation and the immune response (Naka *et al*., 2002). Notably, dysregulation of IL-6 signalling has been shown to be heavily implicated in the development of various chronic inflammatory diseases, including rheumatoid arthritis, Castleman's diease, juvenile idiopathic arthritis and Crohn's disease (Nishimoto & Kishimoto, 2004).

1.3.2 Roles of IL-6

IL-6 is well known for the role it plays in the acute phase response, whereby it potently induces the production of acute phase proteins (Gauldie *et al*., 1987). Examples of acute phase proteins include, C-reactive protein (CRP), fibrinogen, serum amyloid protein and various complement components. The concentration of these proteins increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) by a minimum of 25-fold, the threshold for classification as an acute phase protein, to as much as 1000-fold, as in the case of CRP, in the setting of acute inflammation (Gabay & Kushner, 1999). These fold differences in acute phase proteins have been documented during conditions, such as infection, trauma, surgery, burns, tissue infarction etc. and appear to serve beneficial effects, by initiating, sustaining or modulating the inflammatory process (Gabay & Kushner, 1999). IL-6 is considered to be the chief stimulator of the production of most acute phase proteins (Gauldie *et al*., 1987). The involvement of IL-6 in mediating the acute phase response is demonstrated in studies of IL-6 knock-out mice, whereby this response is severely impaired following tissue damage or infection. Furthermore, these mice display impaired T-cell dependent antibody responses when challenged with virus (Kopf *et al*., 1994).

Epidemiological studies have shown that, in addition to C-reactive protein (CRP), IL-6 plasma levels are strong independent predictors of risk of future cardiovascular events (Rattazzi *et al*., 2003), suggesting an involvement of IL-6 in cardiovascular disease. In more detail, these studies, which included the WHS and WHI (healthy women), PHS (healthy men), and the Iowa65+ Rural Heath (elderly) studies, showed that those subjects in the top quantile of baseline IL-6 levels had

an almost 2 fold greater risk of death and CVD events when compared to those in the lower quantile (Rattazzi *et al*., 2003). Indeed endothelial dysfunction has been shown to correlate with CRP levels (Fichtlscherer *et al*., 2000). Furthermore, a study conducted recently found that patients who were in a stable phase following myocardial infarction displayed a negative correlation between endothelium-dependent vasodilation and IL-6 levels (Erzen *et al*., 2007).

It is interesting to note that IL-6 has been shown to possess anti- as well as pro-inflammatory properties, which appear to depend on the duration of the inflammatory response. For example, during acute responses, IL-6 has been shown to reduce the levels of pro-inflammatory cytokines (Xing *et al*., 1998). However, during states of chronic inflammation, IL-6 becomes pro-inflammatory, involved in the transition from neutrophil to monocyte recruitment in areas of inflammation, a defining feature of the transition from acute to chronic inflammatory states (Hurst *et al.*, 2001; Kaplanski *et al.*, 2003). This may be in part due to increased shedding of the secretory form of the IL-6 receptor, namely sIL-6Rα from neutrophils at sites of acute inflammation, since sIL-6Rα is required for *trans-signalling*, a mechanism described in section 1.3.3. For example, IL-6 and sIL-6Rα have been shown to induce endothelial cells to secrete IL-8 and MCP-1 and upregulate adhesion molecules (Romano *et al*., 1997), establishing an autocrine loop for MCP-1 which favours monocyte recruitment and the transition from acute to chronic inflammation (Marin *et al*., 2001). In contrast, there is a reduction in neutrophil recruitment and an increase in neutrophil phagocytosis, during which levels of TNFα and MCP-1 are upregulated (Gabay, 2006). sIL-6Rα appears to be important in this transition and has been shown to induce the expression of E-selectin, ICAM-1, VCAM-1, IL-8 and IL-6 in human umbilical vein endothelial cells (HUVECs). sIL-6Rα is therefore capable of activating EC inflammation via endothelial-derived IL-6, which can itself be upregulated by IL-6 and sIL-6Rα (Modur *et al*., 1997). Thus, IL-6 appears to modulate the immune response by dictating the recruitment and activation of different leukocyte classes and inducing different cytokines (Jones *et al*., 2005).

As mentioned previously, IL-6 is heavily implicated in the development of chronic inflammatory diseases, such as RA, juvenile idiopathic arthritis, Castleman's disease and Crohn's disease (Nishimoto & Kishimoto, 2004). For example, in

disease models of arthritis, anti-IL-6R antibody has been shown to reduce the development of arthritis (Takagi *et al*., 1998) and interestingly, blockade of sIL-6Rα by a soluble form of the IL-6 receptor (gp130) reduces the severity of arthritis, implicating sIL-6Rα in the development of RA (Nowell *et al*., 2003). Indeed, a humanised anti-IL-6R monoclonal antibody has been used in the treatment of RA with strong therapeutic effects (Nakahara & Nishimoto, 2006). It has also been used in Crohn's disease (Ito *et al*., 2004) and Castleman's disease (Nakahara and Nishimoto, 2006).

1.3.3 IL-6-activated Signalling Cascades

All members of the IL-6 family signal *via* receptor complexes containing the glycoprotein gp130, belonging to the class 1 cytokine receptor family. IL-6, IL-11 and CNTF cannot bind gp130 directly. They first need to bind to their respective membrane-bound α-receptor subunits; IL-6Rα, IL-11Rα and CNTFRα (Davis *et al.*, 1991; Hilton *et al.*, 1994; Yamasaki *et al.*, 1988). Regarding IL-6, endothelial cells do not express IL-6Rα. However, soluble forms of the receptor (sIL-6Rα) exist and these can bind IL-6 to generate a so-called *trans-signalling* sIL-6Rα/IL-6 complex that is capable of efficiently activating gp130. sIL-6Rα can be produced by limited proteolysis (shedding) of membrane-bound receptors from, for example, neutrophils recruited to sites of vascular injury (Marin *et al*., 2002). Additionally, sIL-6Rα can be generated by translation from an alternatively spliced sIL-6Rα transcript (Muller-Newen *et al*., 1996). The remaining IL-6-type cytokines bind directly with their respective signal transducing receptors; LIF, CNTF, CT-1 and CLC bind and signal via heterodimers of gp130 and LIFR, while OSM signals via heterodimers of gp130-LIFR and/or gp130-OSMR (Figure 1.3).

The extracellular domain of all members of the class 1 receptor cytokine family have the characteristic motif CHD_2 mentioned earlier (Bazan, 1990) and different numbers of fibronectin type III (F-III) domains. The CHD_2 of gp130 has been shown to interact with distinct areas on the surface of the IL-6 cytokines. One such site, which is common to all the IL-6 cytokines and binds CHD_2 is site II. In addition to site II, another site termed site III interacts with the second signalling receptor *via* the Ig-like domains of either a second gp130 or LIFR or OSMR. Therefore, even when signalling *via* gp130 homodimers, 2 different sites and 2 different

epitopes on gp130 are required for ligand recognition (Kurth *et al*., 1999). If an α-receptor is involved in signalling, as is the case with IL-6, site I on IL-6 binds with the CHD_2 on sIL-6Rα (Figure 1.4).

- The Janus Kinases (JAKs)

  Most cytokine receptors have no intrinsic tyrosine kinase activity and therefore rely on signalling *via* associated tyrosine kinases. Numerous studies have shown that the JAK family are the predominant kinases utilised by cytokine receptors for this purpose (Muller *et al.*, 1993; Witthuhn *et al.*, 1993). However, another class of tyrosine kinases called the Src family kinases (SFK) have also been shown to be activated by cytokine receptors. Indeed, studies have shown that co-operation between both SFKs and JAKs are required for optimal signal transduction (Ingley & Klinken, 2006). In mammals, the JAK family has 4 members; JAK1, JAK2, JAK3 and TYK2. JAK1, JAK2 and TYK2 are widely expressed, whereas JAK3 is restricted to the haematopoietic immune system (Musso *et al*., 1995). Importantly, JAK3 deficiency is the basis of human autosomal recessive "severe combined immunodeficiency" (SCID) (Macchi *et al*., 1995).

  Structurally JAKs comprise 7 conserved domains, termed The JAK homology (JH) domains 1-7, numbered from the carboxyl to the amino terminus (Figure 1.5 a.). The hallmark of the JAKs is the presence of JH1, which is a functional tyrosine kinase domain, and JH2, which is a catalytically inactive pseudo-kinase domain; the presence of these domains give rise to the kinase's name (the "two-faced" Roman god Janus). Despite lacking tyrosine kinase activity, JH2 is proposed to have regulatory functions. For example, deletion of this domain has been shown to increase JAK2 and JAK3 phosphorylation, as well as signal transducer and activator of transcription (STAT) activation. The JH2 domain is believed to negatively regulate the kinase domain *via* an intramolecular interaction between JH1 and JH2, which effectively suppresses basal kinase activity. Upon ligand binding, conformational changes relieve this interaction and allows activation of JH1 (Saharinen *et al.*, 2000; Saharinen & Silvennoinen, 2002). JH3-JH5 domains have homology with SH2 domains, implying interactions with other signalling components *via* phosphorylated tyrosine residues. However, partners for these domains have yet to be identified (Ingley & Klinken, 2006). Lastly, JH6-JH7 domains constitute a Band 4.1, ezrin, radaxin, moesin (FERM) domain, which has

been implicated in interactions with cytokine receptors (Huang *et al*., 2001), and the JH1 domain to increase activity (Zhou *et al.*, 2001).

The membrane-proximal regions of gp130 predominantly bind JAK1, supported by the findings that in cells lacking JAK1, IL-6 signalling is impaired (Guschin *et al*., 1995). These regions contain conserved motifs called box1 and box2 and both are necessary for efficient JAK binding. Deletions or mutations to box1 result in impaired binding of JAKs to gp130 (Haan *et al.*, 2000; Tanner *et al.*, 1995) and deletion of box2 only leads to JAK association when the kinase is over-expressed (Tanner *et al*., 1995). It has therefore been proposed that box2 increases the affinity of JAK binding (Heinrich *et al*., 2003). In addition to box1 and box2, an interbox1-2 region on gp130 is also involved in JAK binding and again, in studies where this region has been mutated, abrogated JAK signalling has been observed (Haan *et al*., 2000).

Ligand binding causes a conformational change within the gp130 homodimer, which allows JAK transphosphorylation and activation. Following JAK activation, specific tyrosine residues on the receptor become phosphorylated by the activated JAKs. The structural basis of the JAK-receptor interaction and the mechanism by which the receptor re-orientates to receive the phosphorylation is currently very poorly understood. Nevertheless, after the receptor has become phosphorylated at specific tyrosine residues, SH2-domain containing proteins are recruited to the receptor and activate downstream signalling. Two major intracellular pathways activated by gp130 are the signal transducer and activator of transcription (STAT) pathway and the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway.

- The STAT Pathway

  The STAT family of transcription factors were first described by Darnell *et al*. (1994) in the early 90s as ligand-induced transcription factors in cells treated with interferon (IFN) (Darnell *et al.*, 1994; Fu *et al.*, 1992). To date, the STAT family comprises seven mammalian members; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Additionally, alternative splicing of STAT1, 3, 4, 5a and 5b yields isoforms with truncated C-terminal domains, for example the STAT3β isoform lacks the 55 C-terminal amino acids of STAT3α, but gains a unique 7 amino acids (Schaefer *et al*., 1995). The β isoforms have been reported to act as dominant negative regulators of transcription when overexpressed (Caldenhoven

et al., 1996). Moreover, these isoforms have been shown to differ in their transcriptional activities, for example isoform-specific deletions of STAT3β and STAT3α have shown that STAT3β activates a distinct subset of STAT3 genes in response to IL-6 and may not act as a dominant negative *in vivo* (Maritano *et al*., 2004).

STATs are activated by a wide range of cytokines, as well as growth factors and hormones (Lim & Cao, 2006). Depending on the STAT member involved, a range of ligands which are sometimes overlapping can activate STAT1, STAT3, STAT5a and STAT5b, whereas only a few cytokines are capable of activating STAT2, STAT4 and STAT6 (Lim & Cao, 2006). For example, STAT3 can be activated by cytokines and growth factors, including the IL-6 family members and EGF, and is implicated in mitogenesis, survival and anti-apoptosis (Bromberg, 2001). STAT6, on the other hand is predominantly activated by IL-4 and is involved in T helper 2 (Th2) development (Shimoda *et al*., 1996).

Structurally, STATs comprise several distinct functional domains, including an N-terminal domain, a coiled-coil domain, a DNA binding domain, a linker domain, a SH2 domain and a C-terminal transactivation domain (Figure 1.5 b.). The N-terminal domain is involved in dimerisation and tetramerisation, and the recruitment of phophatases for some STATs (Meyer *et al.*, 2004; Ota *et al.*, 2004; Vinkemeier *et al.*, 1998). The coiled-coil domain is implicated in protein-protein interactions. For example, c-Jun has been shown to interact with this domain in STAT3, whereby STAT3 and c-Jun cooperation is required for maximal IL-6-dependent acute-phase response gene activation driven by the α_2-macroglobulin enhancer (Zhang *et al*., 1999b). This domain has also been shown to be involved in receptor binding, whereby mutations within it impair STAT3 recruitment to gp130 (Zhang *et al*., 2000). The DNA-binding domain is highly conserved amongst all the STATs and, as well as binding DNA, it also controls nuclear translocation. It has been proposed to achieve this by maintaining the necessary conformation for importin binding (Ma & Cao, 2006). Importins are required for cytokine-induced nuclear import of STATs, whereas nuclear export involves a “chromosome region maintenance 1” (CRM1)/ exportin1-dependent process (Meyer & Vinkemeier, 2004). The linker domain is implicated in transcriptional activation, since studies using point mutations within this region of STAT1 abolished transcriptional responses to IFN-γ (Yang *et al*., 1999). Additionally, this domain also participates

in protein-protein interactions, demonstrated by STAT3 interaction with "genes associated with retinoid–IFN-induced mortality-19" (GRIM-19), a death regulatory gene product. GRIM-19 association with STAT3 has been shown to block STAT3 transcriptional activity (Liu *et al*., 1998). The most conserved domain within the STATs is the SH2 domain. This domain is essential for binding to receptors *via* specific phospho-Tyr residues (Hemmann *et al*., 1996), and can also mediate dimerisation (Shuai *et al.*, 1994). Lastly, the C-terminal transactivation domain and the conserved tyrosine and serine residues facilitate the activation, dimerisation and transcriptional activation of STATs (Darnell *et al.*, 1994; Shuai *et al.*, 1993). The transactivation domain mediates protein-protein interactions including interactions with the "cAMP response element binding protein" (CREB)-binding protein (CBP) to regulate gene transcription (Gingras *et al.*, 1999; Zhang *et al.*, 1996).

All IL-6 type cytokines are capable of activating STAT1 and STAT3 *via* gp130. However, STAT3 activation has been observed at a greater extent than STAT1 activation (Heinrich *et al*., 1998). STAT recruitment to activated IL-6 type receptors is mediated by the STAT SH2 domain and requires the phosphorylation of certain tyrosine residues. In particular, STAT3 binds four phospho (p)YXXQ motifs of gp130 (Y^{767}RHQ, Y^{814}FKQ, Y^{905}LPQ and Y^{915}MPQ) (Stahl *et al*., 1995), whereas STAT1 is more restricted and binds two (p)YXPQ motifs in gp130 (Y^{905}LPQ and Y^{915}MPQ) (Gerhartz *et al*., 1996). Once recruited, STATs become phosphorylated by JAKs on a single tyrosine residue; Y^{701} in STAT1 and Y^{705} in STAT3 (Kaptein *et al.*, 1996; Shuai *et al.*, 1993). In addition, STAT serine phosphorylation by MAP kinases has also been observed at S^{727} in both STAT1 and STAT3 (Wen *et al.*, 1995). The relevance of this is still not completely understood, but it has been proposed that serine phosphorylation is required for maximal transcriptional activity, since a mutated Ser727 to Ala in STAT3 results in reduced transcriptional activity (Shen *et al*., 2004). Following phosphorylation, activated STATs form homo- and/or hetero-dimer complexes, consisting of STAT1-STAT1, STAT1-STAT3 or STAT3-STAT3 dimers, which translocate to the nucleus to bind response elements of IL-6 inducible genes. STATs bind to essentially 2 types of response elements; (1) interferon stimulated response element (ISRE) and (2) gamma-activated site (GAS). The ISRE appears to be restricted to IFN signalling (Fu *et al.*, 1990), whereas the GAS, including *sis*-inducible element (SIE), acute

phase response element (APRE) and other GAS-like sequences are present in promoters such as *c-fos* and acute phase proteins and are targets of STATs. Target genes downstream of STAT3 include acute phase genes, such as fibrinogen (Wegenka *et al.*, 1993), cell cycle regulators such as cyclin D1, and anti-apoptotic genes such as Bcl-X_L (Bromberg *et al*., 1999).

- ERK1/2 Pathway

  ERK1 and ERK2 are ubiquitously expressed, proline-directed serine and threonine protein kinases, discovered in the early 90s (Boulton & Cobb, 1991) and belong to the mitogen-activated protein kinase (MAPK) family. ERK1 and ERK2 constitute one of three sub-families of MAPK, together with the c-Jun N-terminal kinase subfamily (JNK1, JNK2 and JNK3) and the p38-MAP kinase subfamily (α, β, γ and δ). Additionally, ERK3 - ERK8 have also been identified, but their regulation and roles are much less understood (Bogoyevitch & Court, 2004). ERK1 and ERK2 are generally considered one entity (ERK1/2), since they are 83% identical, with the differences shown to be outside the kinase region (Boulton *et al*., 1991). ERK1/2 is expressed in all tissues and may be activated by a wide range of stimuli, including, growth actors, serum, cytokines, hormones and osmotic stress (Chen *et al*., 2001). ERK1/2 is implicated in many cellular processes, including cell proliferation, survival and differentiation (Kolch, 2005).

  Conventionally, ERK1/2 is activated as the final step in a Raf-MEK-ERK kinase cascade (Figure 1.6). The small G protein Ras is an upstream activator of Raf, and recruits Raf to the plasma membrane. Notably, mutations in Ras genes are found in 30% of all human cancers (Bos, 1989). Raf activation involves phosphorylation at specific Ser, Tyr and Thr residues, which differ between the 3 Raf isoforms; A-Raf, B-Raf and Raf-1/C-Raf. For example, phosphorylation of both Ser338 and Tyr341 appears to be crucial for Raf-1 activation, since mutation of both residues (Ser338/Tyr341 to Ala) has been shown to abrogate Raf-1 activity (Mason *et al*., 1999). Furthermore, phosphorylation of Thr491 and Ser494 has been shown to be necessary, but not sufficient for Raf-1 activation. These sites co-operate with Ser338 and Tyr341 to activate Raf-1 (Chong *et al*., 2001). Alternatively, Ser259 on Raf-1 has been identified as an inhibitory phosphorylation site, whereby this site is phosphorylated in resting cells and requires dephosphorylation to activate Raf-1 or conversely may be hyperphosphorylated by PKA to inhibit Raf-1 activity (Dhillon *et*

al., 2002a; Dhillon *et al.*, 2002b). Thus, activation of Raf-1 involves complex changes in phosphorylation, which are still not fully understood. This complicated activation of Raf-1 also applies to A-Raf. However, B-Raf activation appears to be simpler and this is reflected in the findings that B-Raf activates MEK and ERK more potently than Raf-1 or A-Raf (Galabova-Kovacs *et al.*, 2006; Pritchard *et al.*, 1995). B-Raf has the highest basal activity and has been found to be mutated in many cancers (Repasky *et al*., 2004).

Despite these differences, all 3 Raf isoforms are capable of activating MAP/ERK kinases 1/2 (MEK1/2), except for A-Raf, which cannot activate MEK2 (Beeram *et al*., 2005). MEK1/2 becomes phosphorylated on serine residues 217 and 221 within the activation loop, which is a region within protein kinases that modulate kinase activity (Alessi *et al.*, 1994; Canagarajah *et al.*, 1997). MEK1/2 are dual-specificity kinases that phosphorylate ERK1/2 on Thr (Thr202/185 on ERK1/2 respectively) and Tyr residues (Tyr204/187 on ERK1/2 respectively) (Canagarajah *et al.*, 1997; Owens & Keyse, 2007) in a "TEY" motif within the activation loop. The "TEY" motif is a tripeptide motif including Thr(T)-Glu(E)-Tyr(Y), which depicts the phosphorylation sites of ERK1/2, separated by a single amino acid (Glu). The amino acid between the two phosphorylation sites defines the different groups of MAPK, for example, the aforementioned Glu is the amino acid found in the ERK1, 2 and 5 tripeptide motif, whereas JNKs (JNK1-3) exhibit a Pro (Thr-Pro-Tyr) and p38 MAPKs (α-δ) a Gly (Thr-Gly-Tyr) (Widmann *et al*., 1999). Raf isoforms are the best characterised MEK1/2 activators, however other activators exist, including tumour progression locus 2 (Tpl2) (Salmeron *et al*., 1996).

Structurally, the MAPKs comprise 2 domains; an N-terminal domain which consists of β-sheets and 2 helices, so-called αC and αL16, and the C-terminal domain which is predominantly helical, with 4 short β-strands containing the residues involved in catalysis (Turjanski *et al*., 2007). The catalytic site is localised at the junction between these two domains. MAPKs can be distinguished from other protein kinases by the presence of a 50 residue MAPK insertion in the C-terminal, with an extension of this domain which spans the entire protein. All MAPKs are structurally very similar, which poses the question of how specificity of signalling between the different MAPKs is achieved. Differences in the docking motif binding sites that are located outside the catalytic domain of MAPKs, which dictate substrate specificity could account for this. Furthermore, conformational

changes have been observed in MAPKs upon binding to upstream activators, scaffolds and downstream targets, which can also contribute towards MAPK specificity (Zhou *et al*., 2006).

SHP2 (SH2-domain-containing cytoplasmic protein tyrosine phosphatase) is a ubiquitously expressed and highly conserved enzyme. Like its name suggests, SHP2 comprises two N-terminal SH2 domains and a C-terminal protein tyrosine phosphatase domain and will be detailed in section 1.4.4.1. It is recruited to pTyr759 on gp130 following IL-6 type receptor dimerisation and is subsequently phosphorylated by JAKs. The phosphorylation of SHP2 provides docking sites for the adapter protein Grb2 (growth factor receptor binding protein 2), which is constitutively associated with the GDP/GTP Ras exchange factor, "Son of sevenless" (Sos). It has been proposed that the C-terminal domain residues Tyr542 and Tyr580 within SHP2 interacts with the Grb2-Sos complex (Heinrich *et al*., 2003). Sos recruitment to the receptor complex allows for the activation of Ras, which in turn leads to the activation of the Ras-Raf-MEK-ERK cascade. The activation of ERK1/2 results in the preferential phosphorylation of substrates with the consensus sequence, Pro-Xaa-Ser/Thr-Pro (Gonzalez *et al*., 1991) and more than 150 substrates have been identified (Table 1). In summary, a diagram of the IL-6-activated STAT and ERK1,2 signalling cascades, as described above, is shown in Figure 1.7.

Alternatively, phosphorylated SHP2 has also been shown to associate with the scaffolding proteins Gab1/2 and the p85 subunit of phosphatidylinositol-3-kinase (PI3K), forming a tertiary complex. This complex can go on to activate the Akt pathway and might also feed back into the Ras/ERK cascade (Ernst & Jenkins, 2004).

1.3.4 Leptin

Discovered in 1994 by positional cloning, leptin is the product of the obese (*ob*) gene (Zhang *et al*., 1994). Sequence analysis of leptin showed no strong similarities with any other proteins, however analysis of the tertiary structure using X-ray crystallography, revealed leptin to be a four-helix bundle cytokine, similar to that of the long chain helical family comprising, amongst others, IL-6 (Zhang *et al*., 1997). However, further to this, NMR studies designated leptin a member of the short helix subfamily of cytokines, since the length of the helices were found to be

very similar to those of interleukin-2 (IL-2), interleukin-4 (IL-4) and macrophage-colony stimulating factor (M-CSF), all members of the same short helix family (Kline *et al*., 1997). The receptor, which is encoded by the diabetes (*db)* gene, is termed OB-R and belongs to the same class of receptors as IL-6, namely the class I cytokine receptor family (Tartaglia *et al*., 1995). Thus, while the OB-R and receptors of the class 1 cytokine family, such as gp130, LIFR and G-CSFR share very similar sequences and are classed together on this basis, they do not appear to share the same similarities with respect to their ligands. Despite this, the findings that leptin and OB-R have structural and sequence similarities with other cytokine/cytokine receptor families has greatly aided research into leptin signalling, especially since its discovery has lagged behind that of IL-6, which was discovered in 1986 (Kishimoto, 2006).

Leptin comes from the greek “leptos” meaning thin. As its name suggests, leptin is predominantly known for its role in the regulation of food intake and energy balance. This is clearly demonstrated in the mouse model of leptin deficiency (*ob/ob* mice), which suffers from early onset morbid obesity (Zhang *et al*., 1994). Associated diseases with this model include insulin resistance, diabetes mellitus and infertility (Zhang *et al*., 1994). Thus, leptin’s effects are not simply restricted to body weight and this is further reinforced by the almost universal expression pattern of the leptin receptor in the body. Like IL-6, leptin may therefore be considered a pleiotropic molecule with a range of biological effects.

1.3.5 Leptin Receptors

Leptin acts via transmembrane receptors of which there are at least 6 isoforms; OB-Ra – OB-Rf, encoded by a single gene (Lee *et al*., 1996). All of these splice variants share the same extracellular domain of over 800 amino acids, a transmembrane domain of 34 amino acids (except for OB-Re) and a variable intracellular domain (Figure 1.8) (Beltowski, 2006; Fruhbeck, 2006). The OB-R has 2 CHD_2s and four fibronectin type III domains (Hegyi *et al*., 2004). Based on the different intracellular domains, the OB-R isoforms are classed into (1) short forms (2) long forms and (3) secreted forms. The secreted form of OB-R is OB-Re and is produced by ectoderm shedding of membrane-bound OB-R or alternative splicing (Ge *et al*., 2002). OB-Re contains only the extracellular domain of OB-R and appears to act as a buffering system for free leptin, since the expression pattern of

OB-Re is quite extensive, with levels of OB-Re comparable to those of leptin (Lollmann *et al*., 1997). The short forms of OB-R (OB-Ra, OB-Rc, OB-Rd and OB-Rf), all have the same first 29 intracellular amino acids as the long form, OB-Rb, but only OB-Rb, which has a cytoplasmic domain of 301 amino acids (1162 amino acids full length) has full signalling capability (detailed in signalling section). Indeed, mice deficient in the OB-Rb receptor (*db/db*) display a phenotype characterised by obesity, diabetes mellitus and infertility which is exactly the same phenotype as that of the *ob/ob* mice and mice lacking all the OB-R isoforms (db^{3J}/db^{3J}) (Kowalski *et al*., 2001). Furthermore, this phenotype can be rescued by neuron-specific OB-Rb transgenes (de Luca *et al.*, 2005; Kowalski *et al.*, 2001). This demonstrates the importance of the OB-Rb isoform in leptin signalling and highlights the less understood roles of the short forms of OB-R, despite their distinct tissue distribution (Lee *et al*., 1996). The short forms of OB-R have however, been implicated in the transport of leptin across the blood-brain barrier (Hileman *et al*., 2000), which is supported by the high expression of these short forms in the choroid plexus (Tartaglia, 1997). OB-Rb is predominantly expressed in the hypothalamus (Schwartz *et al*., 1996), but has been found in many tissues, notably in endothelial cells (Sierra-Honigmann *et al*., 1998). A large body of research suggests that OB-Rb is crucial for mediating leptin effects and these effects have been demonstrated to be extremely diverse.

1.3.6 Roles of Leptin

Leptin is mainly produced by white adipocytes. The concentration of circulating leptin has been documented to be relative to body mass index (BMI) and total body fat (Fruhbeck, 2006) and serves to communicate with the CNS to regulate food intake and energy expenditure (Friedman & Halaas, 1998). This role of leptin is heavily supported by the intensely studied mouse models of leptin and leptin receptor deficiency (*ob/ob* and *db/db*), since both models display increased food intake, coupled with decreased energy expenditure. This results in a phenotype resembling morbid human obesity, whereby each mouse model weighs three times more than their normal littermates. Furthermore, treatment of *ob/ob* mice with exogenous leptin was shown to reverse these effects (Ahima *et al*., 1996). On this basis, leptin was initially considered to be a wonder drug for the treatment of obesity. However, it was soon shown that obese individuals actually have elevated

levels of leptin in their bloodstream, due to their increased fat mass, which do not appear to mediate weight loss (Maffei *et al*., 1995). Moreover, leptin administration to these individuals was shown to have minimal effects (Heymsfield *et al*., 1999). This phenomenon gave rise to the concept of leptin resistance occurring at, at least two levels: (1) the level of the blood brain barrier at which the transport system may have become saturated or (2) the level of OB-Rb activation or signalling, whereby the transduction of the leptin signal has become impaired.

Leptin has traditionally been exclusively associated with these central effects on energy homeostasis and body weight. However, like many cytokines, research into leptin has uncovered a wide range of biological activities mediated by this adipokine, whereby involvement in diverse systems, such as the endocrine, immune, reproductive, wound healing, respiratory and the CVS have been observed (Beltowski, 2006; Fruhbeck, 2006; Munzberg *et al.*, 2005; Peelman *et al.*, 2004). Of relevance to the present study are leptin's peripheral effects on the immune system and the involvement of leptin in the pathogenesis of diseases, particularly those of the CVS.

It is well known that obesity is a key risk factor for cardiovascular disease (Van Gaal *et al*., 2006) along with insulin resistance, type 2 diabetes, hypertension and dyslipidemia (Roth, 1997). Hyperleptinaemia may be considered another risk and has been shown to contribute to cardiovascular disease as detailed hereafter. Interestingly, research has shown a link between hyperleptinaemia and endothelial dysfunction, whereby high concentrations of leptin (resembling the concentrations observed in morbid obesity) administered to dogs and rats were shown to attenuate vasodilation of coronary arterioles in response to acetylcholine (Knudson *et al*., 2005). However, studies are conflicting in this area and whether leptin plays a role in endothelial function in humans is currently unknown.

Indeed, it has been proposed that leptin mediates atherogenesis in obese individuals. This is based on the assumption that leptin resistance is selective i.e. only the body weight effects of leptin are impaired in leptin resistance, while other effects are maintained (Beltowski, 2006; Ozata *et al.*, 1999). The concept of selective resistance was suggested by a study using *ob/ob* mice and Agouti yellow obese (Ay) mice, which are mice that have an overexpression of an agouti peptide that blocks melanocortin receptors, leading to obesity. Ay mice exhibit hyperleptinaemia and a higher arterial pressure than their lean littermates.

Interestingly, Ay mice have a higher arterial pressure than *ob/ob* mice, despite having milder obesity than *ob/ob* mice. Thus, it appears that peripheral effects of hyperleptinemia are preserved, whereas central affects are resistant. Administration of exogenous leptin to *ob/ob* mice results in weight loss, however the peripheral effects of leptin administration increase arterial pressure (Correia *et al.*, 2002; Mark *et al.*, 1999). This concept of selective resistance is supported by the findings that *ob/ob* mice on an atherogenic diet are protected from atherosclerosis, despite displaying obesity, diabetes, and hyperlipidemia (Schafer *et al*., 2004). Furthermore, administration of exogenous leptin to these *ob/ob* mice revert from an anti-atherogenic to a pro-atherogenic phenotype and in WT mice exacerbates atherosclerosis, but has no effect in *db/db* mice (Schafer *et al*., 2004). Leptin has also been shown to promote atherosclerosis and thrombus formation in atherosclerosis-prone apoE-deficient mice despite a reduction in adipose tissue mass and fasting insulin levels (Bodary *et al*., 2005). The expression of leptin receptors on various cell types involved in cardiovascular disease, such as monocytes/macrophages (Zarkesh-Esfahani *et al*., 2001) and, importantly, vascular endothelial cells (Bouloumie *et al*., 1998) only strengthens the link between leptin and cardiovascular disease.

In vitro studies have shown that leptin potentiates inflammatory and immune responses by increasing pro-inflammatory cytokines, including TNFα, IL-6 and IL-12 and increasing the phagocytic activity of macrophages (Loffreda *et al*., 1998). Leptin also induces ROS generation and increases expression of MCP-1 in bovine aortic endothelial cells (BAEC) (Yamagishi *et al*., 2001). In addition, leptin has been shown to promote angiogenesis (Park *et al.*, 2001; Sierra-Honigmann *et al.*, 1998) which has been demonstrated to contribute towards unstable plaque growth.

In humans, the *ob* gene mutations are fortunately rare and, in the few reported cases, the phenotype highly resembles that of the *ob/ob* mouse, with individuals displaying the characteristic morbid obesity trait (Montague *et al*., 1997). Of interest, this phenotype has also been associated with low T cell counts and an increased incidence of infectious disease and mortality (Ozata *et al*., 1999). Further to this, (Ciccone *et al*., 2001)have shown an association between plasma leptin levels and insulin resistance and inflammation, each of which are risk factors

for cardiovascular disease (Wannamethee *et al*., 2007). Moreover, in a study conducted by Wolk *et al*. (2004), levels of plasma leptin were demonstrated to predict future cardiovascular events such as cardiac death, myocardial infarction (MI), cerebrovascular accident, or re-vascularization in patients with angiographically confirmed atherosclerosis (Wolk *et al*., 2004). With relation to atherosclerosis, a correlation between the intima-media thickness (IMT) of the common carotid artery, which is an early marker of atherosclerosis, and plasma leptin concentrations has been demonstrated (Ciccone *et al*., 2001). OB-Rb expression has been shown in human atherosclerotic plaques (Park *et al*., 2001) and together with C-reactive protein (CRP), IL-1 and IL-6, leptin has been shown to act as an acute-phase reactant, being produced at high levels during inflammation, sepsis and fever (La Cava & Matarese, 2004).

1.3.7 Leptin-activated Signalling Cascades

Leptin signals *via* OB-R homodimers and mainly activates the JAK/STAT pathway (Vaisse *et al*., 1996) in a very similar way to the IL-6-type cytokine receptors (refer to IL-6 section). In the first instance, leptin binds and causes a conformational change in the OB-Rb homodimer, which enables the transphosphorylation of the OB-Rb-associated JAKs, specifically JAK2 (Kloek *et al*., 2002). JAK2 associates with the OB-Rb via a conserved box1 region (Bahrenberg *et al.*, 2002; Kloek *et al.*, 2002), in comparison to gp130 that has been shown to require both box1 and box2 regions for optimal JAK binding. Since, OB-Rb possesses specific tyrosine residues on its intracellular domain, it is the only isoform with full signalling capability (Hegyi *et al*., 2004). Transphosphorylation and activation of JAK2 following leptin binding leads to phosphorylation of specific tyrosine residues on OB-Rb, providing docking sites for downstream signalling molecules. OB-Rb has 3 conserved tyrosine residues on the intracellular domain; Tyr985, Tyr1077, and Tyr1138 (Tartaglia, 1997). Both Tyr985 and Tyr1138 are phosphorylated upon leptin binding, but Tyr1138 is not (Banks *et al*., 2000).

STAT Pathway

P-Tyr1138 on OB-Rb, serves as a docking site for STATs, since replacement of this residue with serine impairs STAT signalling and results in an obese, hyperphagic phenotype in mice (Bates *et al*., 2003). STAT1, STAT3 and STAT5

have all been demonstrated to bind OB-Rb (Hekerman *et al*., 2005). Despite earlier reports suggesting that Tyr1077 does not participate in leptin signalling due to lack of tyrosine phosphorylation, a study has since shown by the use of point mutations in OB-Rb, that Tyr1077 or Tyr1138 is required for leptin-induced tyrosyl phosphorylation of STAT5, and Tyr1138 is essential for activation of STAT1 and STAT3 (Hekerman *et al*., 2005). Thus, OB-Rb is capable of activating a broader range of STAT proteins than gp130. *In vivo* studies, however have demonstrated that signalling of leptin occurs mainly through STAT3 (Bates & Myers, 2004). Following STAT activation *via* associated JAKs, homo- and/or hetero-dimerisation complexes are formed, which translocate to the nucleus to bind and activate target gene transcription.

ERK1/2 Pathway

SHP2 is recruited to pTyr985 on OB-Rb (Banks *et al.*, 2000; Bjorbaek *et al.*, 2001), becomes Tyr phosphorylated by JAK2 and recruits the adapter protein Grb2 (Banks *et al*., 2000), which then mediates activation of the Ras-Raf-MAPK cascade (mentioned earlier in section 1.3.3) *via* the Ras GEF Sos. In addition, ERK activation has also been observed in the absence of OB-Rb phosphorylation. In this pathway, the short isoform OB-Ra and the OB-Rb lacking all Tyr residues are both able to activate ERK via direct signalling from JAK2 to the ERK pathway. Thus, two pathways of ERK activation are proposed, whereby one pathway requires OB-Rb phosphorylation of Tyr985 and the other pathway does not. However, both pathways require the phosphatase activity of SHP2, since catalytically inactive SHP2 completely inhibits ERK activation. The substrates of SHP2, which mediate ERK activation by leptin have still be identified. Further to this, ERK phosphorylation *via* Tyr985 of OB-Rb requires Tyr phosphorylation of SHP2 (Bjorbaek *et al*., 2001). Refer to Figure 1.9 for a diagram of the leptin-activated STAT and ERK1,2 signalling cascade.

1.4 Regulation of Cytokine Signalling

Cytokine signalling is typically transient, suggesting the involvement of negative regulatory steps aimed at terminating this response. Indeed, controlling these responses is crucial for avoiding detrimental inflammatory outcomes, including the

development of diseases such as atherosclerosis, RA, Crohn's disease and Castleman's disease (Nishimoto & Kishimoto, 2004).

There are many mechanisms with which to negatively control cytokine signalling; these include receptor internalisation, ubiquitin-mediated proteasomal degradation, protein inhibitors and activators of STATs (PIAS), protein tyrosine phosphatases (PTPs) and suppressors of cytokine signalling (SOCS). These endogenous inhibitory mechanisms will be described, with an emphasis on the SOCS proteins as inhibitory regulators of IL-6 and leptin signalling.

1.4.1 Receptor Internalisation

Endocytosis of receptors *via* clathrin-coated vesicles into early endosomes, termed "clathrin-mediated endocytosis" (CME), has three potential consequences; (1) receptors can return to the plasma membrane from where they came (recycling), (2) receptors can be transported to a different domain of the plasma membrane (transcytosis) or (3) receptors can progress to lysosomes, where they are degraded. It is important to note here that receptor endocytosis is no longer solely considered a mechanism for the termination of signalling. Rather, it has also been shown to contribute to cell signalling, whereby signalling processes can occur in endosomes (Miaczynska *et al.*, 2004; Polo & Di Fiore, 2006).

Endocytosis relies on the expression of endocytosis motifs on membrane proteins and their interaction with adapters present in clathrin-coated pits, such as the AP-2 adapter complexes. The most well-studied of these membrane protein motifs include the di-leucine and tyrosine-based motifs (Bonifacino & Traub, 2003). An example of this can be observed in gp130, whereby studies using mutant gp130s with different truncations in the intracellular domain identified a sequence of 10 amino acids (TQPLLDSEER) containing a di-leucine internalisation motif, which was shown to be crucial for receptor- mediated endocytosis of the IL-6 receptor complex (Dittrich *et al*., 1994). However, in addition to these peptide motifs, proteins can be ubiquitinated on cytosolic lysine residues and this can also serve as a signal for endosomal sorting (Bonifacino & Traub, 2003). Unlike cytosolic or nuclear proteins that can be polyubiquitinated and degraded via the proteasome (mentioned later), membrane proteins tend to be modified by mono- or di-ubiquitin conjugates (Belouzard & Rouille, 2006). Certainly, this has been the case with the OB-Ra. The OB-Ra cytoplasmic tail contains no tyrosine or di-leucine-based

endocytosis motifs, but instead possesses two lysine residues which are each ubiquitinated and these act as internalization motifs for clathrin-dependent endocytosis of the receptor (Belouzard & Rouille, 2006).

It is interesting to note that only 5-25% of OB-R isoforms have been located at the cell surface under basal conditions, with the remaining proportion localised to intracellular pools (Barr *et al.*, 1999; Fruhbeck, 2006). Indeed, the fate of OB-Ra and OB-Rb following internalisation is their eventual degradation in lysosomes and this appears to be mediated by a ligand-independent constitutive endocytosis in some studies (HeLa cells) (Belouzard *et al*., 2004) and a ligand-dependent endocytosis of the two OB-R isoforms in other studies (CHO cells) (Uotani *et al*., 1999). OB-Rb, in particular, has been shown to be undergo internalisation to the greatest extent when compared to the other OB-R isoforms, (Barr *et al*., 1999), and this may be a contributory factor to leptin resistance (Fruhbeck, 2006). With regards to gp130, endocytosis has been shown to occur constitutively, independent of IL-6/IL-6R stimulation (Thiel *et al*., 1998).

The mechanisms involved in these intracellular trafficking processes have yet to be fully established. Studies of class 1 cytokine receptor routing are limited, but from the existing studies, all follow the endosomal/lysosomal pathway including the growth hormone receptor (GHR), leptin receptor (LR), prolactin receptor (PRLR), IL-9R and gp130 (Belouzard *et al.*, 2004; Dittrich *et al.*, 1994; Irandoust *et al.*, 2007; Thiel *et al.*, 1998; Uotani *et al.*, 1999), with the exception of the thrombopoietin receptor (TPOR), which is recycled back to the plasma membrane (Royer *et al*., 2005). The lysosomal routing of the G-CSFR from early to late endosomes and lysosomes is of interest, since this process is believed to be mediated by the suppressor of cytokine signalling 3 (SOCS3) protein *via* ubiquitination of G-CSFR (Irandoust *et al.*, 2007). SOCS3 will be discussed in more detail in section 1.6.5 and represents another inhibitory mechanism of cytokine signalling. Thus, the lysosomal routing of G-CSFR depicts a novel mechanism of inhibition, involving two pathways, i.e. the endosomal/lysosomal pathway and SOCS3.

Further to CME, clathrin-independent mechanisms of endocytosis also exist, such as raft/caveolae-mediated endocytosis (RCE), which is a much less studied endocytotic mechanism than the former. It is of interest because caveolae are especially abundant in specific cell types like endothelial cells (Couet *et al*., 2001).

Caveolae can be described as small, omega-shaped domains enriched in cholesterol, sphingolipids and caveolin proteins. Such domains have been implicated in cell signalling processes as well as endocytosis (Couet *et al*., 2001). Notably, studies have demonstrated the involvement of these lipid rafts in IL-6-mediated STAT activation, whereby disruption of the rafts by methyl-β-cyclodextrin, which removes cholesterol from the membrane, inhibits STAT signalling (Sehgal *et al*., 2002). Caveolin-1 has also been shown to modulate insulin signalling, EGF signalling and, importantly, cAMP signalling (Abulrob *et al.*, 2004; Nystrom *et al.*, 1999; Razani *et al.*, 1999).

Thus, CME and RCE can either dictate receptor fate or modulate cell signalling pathways. Which of these endocytic pathways are used and under what conditions is a question that still remains to be answered. The choice of pathways could potentially be influenced by the type of stimulus; for example IL-6 treatment has been shown to increase trafficking of TGF-β1 receptors to non-lipid raft-associated pools and this leads to enhanced TGF-β1-Smad signalling (Zhang *et al*., 2005). Alternatively, it could be the ligand concentration, since the EGFR has been shown to be internalised almost exclusively via the CME pathway at low doses of EGF, and the RCE pathway at high doses of EGF (Sigismund *et al*., 2005). Overall, endocytic pathways appear to play a very complex role in the integration and attenuation of signals and could potentially have effects on IL-6- or leptin-mediated signalling cascades in endothelial cells.

1.4.2 PIAS

Mammalian “protein inhibitor of activated STAT” (PIAS) proteins, comprise PIAS1, PIAS3, PIASx (PIAS2) and PIASγ (PIAS4), each existing as 2 isoforms, except for PIAS1. As their name suggests, PIAS proteins were originally identified as negative regulators of STAT proteins, (Chung *et al.*, 1997; Liu *et al.*, 1998). Despite their nomenclature, it has now emerged that PIAS proteins are capable of positively or negatively regulating a broad range of proteins. Examples of the proteins that can be regulated by PIAS proteins, which are involved in immune regulation, are shown in (Table 2).

Every member of the PIAS family has been shown to regulate STAT signalling (Shuai & Liu, 2005). Notably, PIAS1 and PIAS3 have been shown to interact with STAT1 and STAT3 respectively (Chung *et al.*, 1997; Liu *et al.*, 1998). PIAS-STAT

interactions are cytokine-dependent and result in inhibition of STAT-mediated transcription. Transcriptional inhibition can result from PIAS proteins blocking the DNA-binding activity of transcription factors. For example, PIAS1 and PIAS3 have both been shown to inhibit the DNA-binding activity of STAT1 and STAT3 respectively (Chung *et al.*, 1997; Liu *et al.*, 1998). The mechanism by which this occurs however is still unclear. Alternatively, PIAS proteins can also recruit co-regulators, such as histone deacetylases (HDACs), which repress transcription. For example, PIASx inhibits IL-12-mediated STAT4 activation *via* recruitment of HDACs (Arora *et al*., 2003). Interestingly, PIAS proteins have also been shown to act as E3 ligases for "small ubiquitin-related modifier" (SUMO) (Kotaja *et al*., 2002). SUMO is known as an ubiquitin-like protein (ULP) because of its similarity with ubiquitin (described in the next section). Sumoylation involves the conjugation of SUMO to protein substrates: for example, sumoylation of STAT1 by PIASx-alpha *via* Lys703 has been observed. However the functional consequences of this modification have yet to be determined, since it does not appear to alter transcriptional activation (Rogers *et al*., 2003). Thus, the role of PIAS-mediated sumoylation in the context of STAT signalling is unclear. However, in general, sumoylation has been implicated in many cellular processes including, the modulation of transcription factors, the targeting of proteins to the nucleus, protein-protein interactions and protein stability (Johnson, 2004).

1.4.3 Ubiquitin-mediated Proteasomal Degradation

Protein degradation in mammalian cells is predominantly mediated by the ubiquitin-proteasome system (UPS) (von Mikecz, 2006), and is crucial in many cellular events, including antigen presentation (Kloetzel, 2001), cell cycle progression (Koepp *et al*., 1999) and removal of misfolded proteins (Hilt & Wolf, 1996). Ubiquitination primarily involves targeting proteins for degradation *via* the 26S proteasome, but has also been implicated in endocytosis (Reggiori & Pelham, 2001; Strous *et al.*, 1996) and cell signalling (Khush *et al.*, 2002; Ting & Endy, 2002).

The process of ubiquitination involves a series of enzymatic steps, beginning with the activation of ubiquitin (Ub) *via* formation of a thioester bond between an ubiquitin (Ub)-activating enzyme (E1) and the carboxyl group of Gly76 on Ub. The activated Ub is then transferred to an Ub-conjugating enzyme (E2), before transfer

from E2 to the substrate lysine residue by E3 ubiquitin ligase. Thus, 3 enzymes are involved in the ubiquitination of substrates, which ultimately results in the formation of an iso-peptide linkage between Lys residues on the substrate and Gly76 on Ub. Although 3 enzymes are involved in this process, only the E3 dictates specificity for the target protein and acts to ubiquitinate the substrate by either accepting the Ub from E2 and transferring it to the substrate or by bringing the substrate into close proximity with the E2 ligase-activated Ub complex (Pickart, 2001; Walters *et al.*, 2004). Substrates can either be monoubiquitinated or polyubiquitinated at one or multiple sites. Polyubiquitin chains are formed by the addition of more Ub moieties to existing Ub *via* linkage between specific Lys residues in Ub and the C-terminal residue Gly76. These linkages to Lys residues appear to dictate the fate of the protein substrate. For example, linkage at Lys48 typically targets substrates to the 26S proteasome (Thrower *et al*., 2000). In contrast, linkage at Lys63 has been implicated in nonproteolytic signalling, for example activation of IκB kinase (IKK) in the NF-kappaB signalling pathway (Deng *et al*., 2000).

Two major types of E3 ubiquitin ligases exist, defined by the presence of either a "homologous to E6-associated protein C terminus" (HECT) domain or a "really interesting new gene" (RING) fold. Recently, a new class of E3 ligase has been described, which contains a Lin11/Isl-1/Mec-3 (LIM) domain. The function of the LIM domain, which is present in many proteins remains largely unknown, although involvement in protein-protein interactions has been suggested (Dawid *et al*., 1998). Importantly, a LIM domain protein known as STAT-interacting LIM protein (SLIM) has been shown to act as a ubiquitin E3 ligase and targets Tyr-phosphorylated STAT1 and STAT4 for proteasomal-mediated degradation. Overexpression of SLIM results in impaired STAT1 and STAT4 activity due to decreased STAT protein levels. Furthermore, deficiency of SLIM in T cells leads to increased levels of phosphorylated and total STAT4, and increased amounts of IFNγ when stimulated with IL-12, in keeping with the major role of IL-4 signalling in the differentiation of IFNγ-secreting Th1 cells (Tanaka *et al*., 2005).

1.4.4 PTPs – a focus on SHP2

Protein phosphatases reverse the effects of protein kinases by catalysing the removal of phosphoryl groups. In terms of signal transduction, dephosphorylation

of proteins can alternatively initiate, sustain or terminate signals (Andersen *et al*., 2001). Protein tyrosine phosphatases (PTPs) comprise a large family of proteins, which are characterised by a unique signature motif. Residues in this motif form the phosphate-binding loop and two residues in particular, namely Cys and Arg, are critical for the catalytic activity of PTPs (Andersen *et al.*, 2001; Tiganis & Bennett, 2007). PTPs can be grouped into two general families; (1) the tyrosine-specific PTPs, which can dephosphorylate substrate proteins on tyrosine; these can be further sub-divided into transmembrane, receptor-like PTPs and non-transmembrane PTPs, and (2) the dual-specificity phosphatases (DSPs), which can dephosphorylate protein substrates on tyrosine, serine and threonine residues (Tiganis & Bennett, 2007). Our understanding of PTPs is greatly lagging behind that of PTKs, which is partly due to the discovery of PTKs a decade before PTPs. PTPs exhibit a high degree of specificity for their substrates. This is achieved by the PTP catalytic domain, which recognises the specific phosphorylated residues and the flanking amino acids within the substrate, and the non-catalytic N- and C-terminal domains, which target the PTP to particular intracellular compartments for substrate recognition (Andersen *et al*., 2001). PTP specificity can be demonstrated by the subfamily of DSPs, termed the mitogen-activated protein kinase phosphatases (MKPs), which dephosphorylate MAPKs on tyrosine and threonine residues. Of the 10 members that make up this family, some can specifically target one class of MAPK (e.g. DUSP6/MKP-3 which specifically dephosphorylates ERK) while others can target more than one class of MAPK (e.g. DUSP1/MKP-1 which dephosphorylates ERK, JNK and p38 MAP kinases) (Owens & Keyse, 2007).

The prototypical PTP is PTP1B which was discovered in 1988 (Charbonneau *et al*., 1988). It has been shown to have numerous substrates, but the most extensively studied of these include the insulin receptor (IR) and JAK2 (Tiganis & Bennett, 2007). A lot of this information has come from the study of PTP1B-deficient mice, which exhibit enhanced insulin sensitivity, which is associated with increased tyrosine phosphorylation of the insulin receptor in muscle and liver. Furthermore, these mice are resistant to diet-induced obesity (Elchebly *et al*., 1999). Further studies have revealed the involvement of leptin signalling in the above phenotype and have demonstrated PTP1B inhibition of leptin signalling *via* dephosphorylation of JAK2 (Cheng *et al*., 2002).

T-cell-specific protein tyrosine phosphatase (TCPTP), including its nuclear isoform TC45, has several proposed substrates, including the IR, EGFR (Tiganis & Bennett, 2007), JAK1, JAK3 (Simoncic *et al*., 2002) STAT1 (ten Hoeve *et al*., 2002) and STAT3 (Yamamoto *et al*., 2002). Of interest, Yamamoto *et al*. (2002) demonstrated TC45-mediated suppression of STAT3 activation in response to IL-6 in 293T cells, implicating the nuclear isoform of TCPTP in the negative regulation of IL-6 signalling. This negative regulation is supported by studies of TCPTP-deficient mice. These mice display a complex phenotype in comparison to PTP1B-deficient mice, wherein the mice are viable but exhibit haemopoietic defects, resulting in splenomegaly, lymphadenopathy and thymic atrophy. As a result, the mice die at 3-5 weeks. Specifically, homozygous mice display defects in bone marrow, B cell lymphopoiesis, and erythropoiesis, as well as impaired T and B cell functions. Taken together, the abnormalities displayed in TCPTP-deficient mice strongly suggest a crucial role of TCPTP in hematopoiesis and immune function (You-Ten *et al*., 1997).

1.4.4.1 SHP-2

The SH2-domain containing protein tyrosine phosphatases (SHPs) are a subfamily of non-transmembrane PTPs comprising two vertebrate SHPs, SHP1 and SHP2. SHP1 expression is restricted to cells of the haematopoietic system, whereas SHP2 is ubiquitously expressed. Both proteins contain two N-terminal SH2 domains (N-SH2 and C-SH2) and a C-terminal catalytic phosphatase domain. As such, SHP1 and SHP2 have the unique ability to function as phosphatases, dephosphorylating signalling components and down-regulating signal transduction, whilst also serving as adapter molecules *via* their SH2 domains, recruiting further adapter molecules to transducer downstream signalling (Heinrich *et al.*, 2003; Neel *et al.*, 2003; Salmond & Alexander, 2006). SHP1 and SHP2 appear to have non-redundant roles, since deletion of either protein in mice results in death at 2-3 weeks due to severe inflammation, so-called the “motheaten” phenotype because of the patchy hair loss caused by sterile dermal abscesses (SHP1), (Neel *et al*., 2003) or embryonic lethality due to defective gastrulation or mesodermal differentiation (SHP2) (Neel *et al*., 2003). Thus, one SHP does not compensate for the other in these phenotypes. The differences between SHP1 and SHP2 effects appear to be due to the differences in SH2 domain-mediated protein interactions,

as well as differences in the PTP domains of both proteins (Salmond & Alexander, 2006).

Of particular relevance to the present study is the finding that SHP2 binds to Tyr759 on gp130 and Tyr985 on OB-Rb (Carpenter *et al.*, 1998; Stahl *et al.*, 1995). The dual function of SHP2 as a phosphatase and an SH2 domain-containing protein poses the question; does SHP2 serve as a positive or negative regulator of IL-6 or leptin signalling?

1.4.4.2 Positive Effects of SHP2 on the ERK Pathway

The finding that ERK activation is inhibited in mice with a mutation of Tyr759 to Phe ($gp130^{F759/F759}$) or cells transfected with a mutated Tyr759 gp130 construct, establishes a positive regulatory role of SHP2 on the ERK pathway (Kim & Baumann, 1999; Ohtani *et al.*, 2000). Furthermore, catalytically inactive dominant-negative SHP2 mutants have been shown to block leptin-mediated ERK phosphorylation and ERK-dependent gene transcription from the egr-1 promoter, suggesting a positive role of SHP2 in leptin signalling (Bjorbaek *et al*., 2001). Indeed, previous studies on growth factor signalling, including EGF, insulin and platelet-derived growth factor (PDGF) signalling have each demonstrated a positive regulatory role of SHP2 on ERK signalling and gene expression (Bennett *et al.*, 1994; Bennett *et al.*, 1996; Yamauchi *et al.*, 1995).

1.4.4.3 Positive and Negative Effects of SHP2 on the STAT Pathway

In contrast to these positive effects, $gp130^{F759/F759}$ mice and cells transfected with a Tyr759→Phe mutated gp130 construct have demonstrated impaired SHP2 activation, prolonged STAT3 and STAT1 activation and enhanced acute-phase protein gene induction, suggesting a negative role of SHP2 on the STAT pathway (Ohtani *et al.*, 2000; Schaper *et al.*, 1998). In further support of this negative regulatory role, the $gp130^{F759/F759}$ mouse phenotype displays splenomegaly, lymphadenopathy and an enhanced acute phase reaction (Ohtani *et al*., 2000). However, these results are complicated by the findings that suppressor of cytokine signalling 3 (SOCS3), another inhibitory mechanism of cytokine signalling detailed in the next section, binds to the same site as SHP2 on gp130 and OB-Rb (De Souza *et al*., 2002) and could therefore contribute towards these negative effects. To address this issue, studies have employed catalytically inactive dominant

negative SHP2 mutants in gp130 signalling. Expression of these mutants have resulted in increased gp130, JAK and STAT3 phosphorylation as well as gene induction (Lehmann *et al.*, 2003; Symes *et al.*, 1997), which confirms the involvement of SHP2 in the negative regulation of the STAT pathway *via* gp130.

These negative effects of SHP2 on gp130 signalling do not appear to be observed for OB-Rb signalling, since mutation of Tyr985 of OB-Rb has no effect on STAT activation (Li & Friedman, 1999). Also, using dominant negative SHP2 strategies (COS-1 cells), SHP2 was shown not to have an effect on STAT3 phosphorylation or STAT3-mediated gene transcription from the SOCS3 promoter (Bjorbaek *et al*., 2001). However, these studies demonstrated no effect of SHP2 on STAT activation and STAT-dependent promoter activity following 15 minutes and 6 hours of leptin treatment respectively. Further research by this group has demonstrated that following 24 hours of leptin treatment, STAT-mediated transcription was enhanced in cells expressing mutated Tyr985Phe OB-Rb (Bjorbaek *et al*., 2001) and suggested that the induction of SOCS3 by leptin over prolonged leptin treatment could account for the enhanced STAT3 response, implicating SOCS3 involvement and not SHP2. In addition, over these extended periods of leptin treatment, SHP2 could possibly act as an indirect positive regulator of the STAT pathway, preventing SOCS3 binding to the OB-Rb at Tyr985 (Bjorbaek *et al*., 2001).

1.4.4.4 Possible Mechanisms of SHP2's Actions on ERK and STAT Signalling

Thus, in general, a positive role of SHP2 in cytokine-induced ERK activation and a negative or positive role of SHP2 in cytokine-induced STAT activation have been proposed. How SHP2 mediates this positive effect on ERK activation and the contribution of SHP2 to the negative regulation of STAT signalling, in relation to SOCS3, are still areas under investigation. To address the first point, SHP2 appears to exert a positive effect on ERK signalling by acting as an adapter protein, wherein SHP2 becomes recruited to phosphotyrosine residues on activated receptors and following activation, associates with the adapter protein Grb2, which is bound to the Ras GDP-GTP exchange factor Sos. This Grb2/Sos complex can then go on to activate the Ras-ERK pathway (as discussed earlier in the signalling sections). This has been demonstrated for growth factor receptor signalling, including FGFR, and OB-Rb (Banks *et al.*, 2000; Bennett *et al.*, 1994; Li

et al., 1994; Myers, 2004). With regards to gp130 signalling, it has been proposed that the SHP2-Grb2 mode of ERK activation could be adopted (Heinrich *et al*., 2003). However, in addition to this, Grb2-associated binder-1 (Gab1) has been shown to become tyrosine phosphorylated in response to IL-6 and associates with SHP2 and PI3K to activate ERK (Takahashi-Tezuka *et al*., 1998). Alternatively, SHP2 can act as a Tyr phosphatase, dephosphorylating particular substrates, which are negatively regulated by tyrosine phosphorylation. For example, SHP2 has been shown to dephosphorylate tyrosine residues on EGFR required for RasGAP recruitment to the EGFR, thereby inhibiting phosphorylation dependent translocation of RasGAP to the plasma membrane and maintaining Ras and hence ERK activation (Agazie & Hayman, 2003).

A model of SHP2 phosphatase activity exists, whereby the binding of SHP2 to phosphotyrosine residues has been shown to activate its phosphatase activity (Barford & Neel, 1998). This can occur in two ways, (1) the SH2 domains can bind to pTyr motifs on activated receptors, such as pTyr759 on gp130, which leads to unfolding of the protein and subsequent phosphatase activation or (2) the SH2 domains can bind to pTyr542 and 580 on the C-terminal tail of SHP2 itself and cause conformational changes leading to activation. If pTyr binding does not occur, SHP2 remains in an inactive state, whereby the N terminal SH2 (N-SH2) domain appears to sterically hinder the access of phosphotyrosine substrates to the PTP domain, as demonstrated by its crystal structure (Hof *et al*., 1998). Therefore, the N-SH2 domain of SHP2 can either bind and inhibit the phosphatase, or bind to phosphotyrosines to activate the enzyme (Figure 1.10). Interestingly, in the human autosomal dominant disorder Noonan syndrome (NS), approximately 50% of all cases are caused by mutations in the SHP2 gene, *PTPN11*, and specifically in portions of the amino N-SH2 domain. These mutations lock SHP2 in its active conformation and subsequently cause excessive SHP2 activity (Tartaglia *et al*., 2001). NS is characterised by short stature, cardiac defects, facial dysmorphia and an increased risk of developing leukaemia (Salmond & Alexander, 2006). Of relevance, this syndrome displays aberrant regulation of the Ras/ERK pathway (Bentires-Alj *et al*., 2006). Thus, NS demonstrates an involvement of SHP2 on ERK activation in humans. However, further research is required to better understand the exact mechanisms involved.

Likewise, the mechanisms by which SHP2 negatively or positively regulates STAT activation are not well understood and the relative contribution of both SHP2 and SOCS on gp130 or OB-Rb signalling is still unclear. There are reports showing that SHP2 can act directly as a STAT phosphatase (STAT5a) (Chen *et al.*, 2003; Yu *et al.*, 2000) and indeed STAT3/SHP2 complexes have been detected (Gunaje & Bhat, 2001). A large body of evidence supporting a negative role of SHP2 in gp130-mediated STAT activation exists, whereas conversely, SHP2 has been shown to have no effects on OB-Rb-mediated STAT3 phosphorylation (Bjorbaek *et al*., 2001). The possibility that SHP2 acts as an indirect positive regulator of STAT3, impeding SOCS3 recruitment at the Tyr985 site, has been described (Bjorbaek *et al*., 2001). Clearly, the involvement of SHP2 in gp130 and OB-Rb signalling requires further study.

1.4.5 SOCS Family of Proteins

There are eight members of the suppressor of cytokine signalling (SOCS) family of proteins to date; CIS (cytokine-inducible SH2 domain-containing protein) and SOCS1 through to SOCS7. SOCS1 was the first member to be discovered in 1997 by three independent groups (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). Using the predicted amino acid sequence of SOCS1 as a probe, database searches identified 20 proteins with shared sequence homology within the C-terminal SOCS box region. Based on the presence of a central SH2 domain, the SOCS proteins were subdivided into a group of their own. The remaining proteins were divided into the following groups; WD-40-repeat proteins with a SOCS box (WSB proteins), ankyrin repeat proteins with a SOCS box (ASB proteins), sprouty (SPRY) domain-containing SOCS box proteins (SSB proteins) and GTPase domain-containing proteins (RAR and RAR-like proteins) (Krebs & Hilton, 2001). In addition to a central SH2 domain, all members of the SOCS family contain an amino-terminal of variable length (50-380 amino acids) and a conserved 40 amino acid carboxyl terminal SOCS box (Alexander, 2002; Yoshimura *et al.*, 2007). Three systems of nomenclature exist for the SOCS proteins, but the SOCS nomenclature is the most widely accepted. Further analysis of the primary amino acid sequences of all SOCS members revealed paired associations according to sequence similarity. Thus, CIS and SOCS2, SOCS1 and SOCS3, SOCS4 and SOCS5, and SOSC6 and SOCS7 form related pairs. CIS, SOCS1, SOCS2 and

SOCS3 are quite well characterised, while the remaining members are poorly understood in comparison. Since SOCS1 and SOCS3 are well studied, homology-paired and have been shown to potently inhibit IL-6/leptin signalling, focus will be placed on these SOCS members and on SOCS3 in particular.

1.4.5.1 SOCS Proteins as Inhibitors of Cytokine Signalling

SOCS proteins function as classical negative feedback inhibitors of cytokine signalling, since most SOCS proteins are themselves induced by cytokines. Cytokines shown to induce SOCS include the gp130 signalling cytokines, IL-2, IL-3, IL-4, IL-10, IFN-γ, G-CSF and leptin. (Alexander, 2002; Yoshimura *et al.*, 2007). Other inducers of SOCS proteins comprise Toll-like receptor (TLR) agonists (e.g. LPS, CpG-DNA), GH, prolactin, statins and importantly, cAMP (Dalpke *et al.*, 2001; Gasperini *et al.*, 2002; Lang *et al.*, 2003; Yoshimura *et al.*, 2007). The SOCS proteins can inhibit signalling by multiple mechanisms according to the SOCS member and signalling pathway involved. Currently, there are 3 known mechanisms by which SOCS proteins can downregulate cytokine signalling (Figure 1.11):

1. SOCS proteins can bind specific pTyr residues via their SH2 domain. SOCS3 binds to the pTyr motif 759 (pTyr759/mouse homologue pTyr785) on gp130 (Nicholson *et al*., 2000) and pTyr985/Tyr1077 on the leptin receptor (Eyckerman *et al*., 2000) and physically occupies the same sites as other SH2 domain-containing signalling components, such as SHP2, thereby competing with and subsequently inhibiting other signalling pathways (De Souza *et al.*, 2002; Heinrich *et al.*, 2003). Peptide studies have shown that the binding specificity of SOCS3 is very similar to that of SHP2, with optimal SOCS3 and SHP2 phosphopeptide ligands containing overlapping consensus sequences (De Souza *et al*., 2002). The same group showed that SOCS3 binds to the gp130 receptor with much higher affinity than the leptin receptor (De Souza *et al*., 2002). However, the findings that SOCS3 can bind two sites on the leptin receptor may compensate for the low affinity each site exhibits for SOCS3, for example two SOCS3 molecules are capable of binding the leptin receptor simultaneously, whereas only one SOCS3 molecule can bind gp130 at any one time (De Souza *et al*., 2002).

2. The kinase inhibitory region (KIR) of SOCS1 and SOCS3, located downstream of the SH2 domain, is capable of interacting with the substrate binding site of the kinase domain in JAK2, acting as a pseudosubstrate and thus inhibiting the catalytic activity of JAK2 and activation of signalling from the associated receptor (Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999). Specifically, Tyr31 of SOCS3 and Tyr65 of SOCS1 have been identified as the residues responsible for the pseudosubstrate inhibition of JAK2 (Bergamin *et al*., 2006). Interestingly, structural data relating to this interaction has revealed that it is implausible for Tyr31 or Tyr65 to reach the active kinase domain of JAK2 whilst bound *via* the SH2 domain i.e. in *cis* (Bergamin *et al*., 2006). This does not rule out the possibility that the SOCS proteins could bind to one JAK via their SH2 domain and inhibit another JAK via pseudosubstrate inhibition i.e. in *trans,* or the possibility that binding of the SOCS SH2 domain to the specific phosphotyrosine residues as outlined above positions the KIR for binding to the kinase domain of associated JAK2. This appears to be a more likely scenario than the former *trans* concept, since the crystal structure of the SOCS3/gp130 and various structural data favour the physiological target of SOCS3 SH2 domain to be pTyr757/759 of mouse/human gp130 and not the activation loop of JAK2 (Bergamin *et al*., 2006).

3. The SOCS box present within all SOCS members can recruit elongins B and C, which together with cullin 5 and RING-box 2 (Rbx2) form an E3 ubiquitin-ligase complex. This complex associates with enzymes E1, a ubiquitin-activating enzyme and E2, a ubiquitin-conjugating enzyme, to mediate Lys48 polyubiquitination and subsequent proteasomal degradation of signalling components bound to the SOCS proteins via their SH2 domains (Kamura *et al.*, 2004; Ungureanu *et al.*, 2002; Zhang *et al.*, 1999a). A possible ubiquitination site, Lys-6 is also present at the N-terminus of SOCS3. A truncated isoform of SOCS3 lacking this site has a much longer half-life than the wild-type SOCS3, suggesting that Lys-6 plays an important role in proteasomal degradation. Moreover, this demonstrates that SOCS3 expression can be regulated at a post translational level (Sasaki *et al*., 2003).

Ubiquitin-mediated degradation has been proposed to be quite a generic mechanism of degradation, since SOCS proteins can potentially target the whole receptor-cytokine complex including the JAKs, plus the SOCS proteins themselves

for proteasomal degradation by the proteasome. It poses the question of how SOCS proteins selectively block JAK signalling at one type of receptor and leave other receptors using the same JAKs? This could possibly be explained by using the former concept mentioned in 2 above i.e. that SOCS SH2 domains could be preferentially binding the specific phosphotyrosine residues on activated receptors rather than JAKs, thereby causing degradation of associated JAKs as well as the receptor-cytokine complex, and achieving specifity at the receptor level. Indeed, it has been shown that mutation of Tyr757 to Phe on murine gp130 is sufficient to cause enhanced IL-6-inducible gene expression (Anhuf *et al*., 2000). Furthermore, bone marrow-derived macrophages (BMDM) isolated from mice with a mutation of Tyr757 to Phe in gp130 is sufficient to switch the IL-6 mediated response to an 'IL-10-like' anti-inflammatory response, in terms of inhibiting LPS-induced induction of pro-inflammatory cytokines (El Kasmi *et al*., 2006). Previous studies have linked the absence of SOCS3 with the establishment of the anti-inflammatory response following IL-6 treatment (Yasukawa *et al*., 2003) as will be discussed later, so the former data suggests that mutation of only the specific phosphotyrosine that binds SOCS3 is sufficient to cause cytokine receptors to become refractory to SOCS inhibition, despite the presence of JAKs. Specific phosphotyrosine binding of SOCS members therefore appears to be the main mode of SOCS3 association with the receptor and not JAK association *via* the SH2 domain. This is in contrast to SOCS1, since studies have shown that the phenotype of SOCS1- deficient mice can only be partially rescued in mice with SOCS1 lacking the SOCS box, but retaining the SH2 domain. This shows that both the SOCS box and SH2 domain are required for the inhibitory effects on IFN-γ signalling (Zhang *et al*., 2001).

In contrast to the above findings of SOCS interaction with elongins B and C, leading to proteasomal degradation, some studies have found that interaction with the elongin BC complex can stabilise SOCS3 (Haan *et al*., 2003) and SOCS1 (Kamura *et al*., 1998). Haan *et al*. (2003) showed that tyrosine phosphorylation of SOCS3 disrupted elongin interaction, which accelerated SOCS3 degradation. This may suggest that tyrosine phosphorylation of SOCS3 is a prerequisite to proteasomal degradation. Indeed, Haan *et al*. (2003) suggested that the elongin BC interaction with SOCS3 may function to associate SOCS3 with a latent ubiquitination complex that only becomes active when SOCS3 is phosphorylated.

SOCS phosphorylation causes the dissociation of elongin C and the bringing together of the ubiquitination machinery into close proximity with SOCS3, subsequently triggering its degradation (Haan *et al*., 2003). This concept therefore combines the both views of elongin BC interaction and proteasomal degradation.

1.4.5.2 SOCS Proteins as Regulators of Other Signalling Pathways

In addition to the involvement of SOCS proteins in cytokine signalling, SOCS1 and SOCS3 have been shown to bind both EGF and FGF receptors (EGFR, FGFR) and affect downstream signalling events, in both positive and negative ways (Ben-Zvi *et al.*, 2006; Xia *et al.*, 2002). With regards to EGF signalling, SOCS1 and 3 have been shown to facilitate EGFR proteasomal degradation in HEK293 cells (Xia *et al*., 2002), while SOCS1 has been shown to inhibit STAT1 phosphoryation, but elevate ERK phosphorylation in response to FGF treatment in rat chondrosarcoma (RCS) cells (Ben-Zvi *et al*., 2006). Furthermore, SOCS1 and SOCS3 have been demonstrated to associate with insulin receptor substrate 1 (IRS1) and IRS2 following insulin stimulation and interact with the elongin BC ubiquitin-ligase complex to promote Lys48 polyubiquitination and degradation (Rui *et al*., 2002).

As mentioned earlier, SOCS3 offers another level of regulation by being able to become tyrosine phosphorylated itself on residues 204 and 221 located in the SOCS box by IL-2, erythropoietin (EPO), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Cacalano *et al*., 2001). The phosphorylated tyrosine residue 221 has also been shown to associate with and inhibit RasGAP. Using a murine B cell line, it was found that WT SOCS3 could inhibit IL-2-mediated STAT5 phosphorylation, but maintain IL-2-mediated ERK phosphorylation, whereas a Tyr204/221Phe mutant SOCS3 still inhibited STAT5 phosphorylation, but in contrast to the WT, abolished ERK phosphorylation, suggesting a phosphorylation-dependent maintenance of ERK signalling. (Cacalano *et al*., 2001). This inhibitory effect of the mutant was also observed following EPO and PDGF treatment. Thus, phosphorylation of Tyr204 and Tyr221 of SOCS3 following growth factor stimulation leads to pTyr221 interaction with the SH2 domain of RasGAP, which subsequently sustains the activation of Ras and ERK. The duration of ERK signalling has been shown to be important for determining biological outcome, for example sustained activation of ERK has been

shown to be required for the control of G1 progression by regulating cyclin D1 activation (Weber *et al*., 1997). SOCS3 therefore appears to have pathway-specific effects as well as receptor-specific effects, and appears to show positive regulation as well as its well-known negative regulation, adding further complexities to its actions.

Another level of complexity demonstrated by SOCS proteins is their ability to interact with other SOCS family members (Piessevaux *et al.*, 2006; Tannahill *et al.*, 2005). For example, although SOCS2 plays a major role in the negative regulation of GH signalling (Greenhalgh *et al*., 2005), it has also been shown to enhance GH signalling. This is believed to be caused by the binding of SOCS2 to other SOCS members and modulating their activity *via* the elongin BC complex, with subsequent proteasomal degradation (Piessevaux *et al.*, 2006; Tannahill *et al.*, 2005). This SOCS2-mediated inhibitory effect on other SOCS members has been observed on SOCS1- and SOCS3-dependent inhibition of growth hormone (GH) signalling, thus potentiating GH signalling (Piessevaux *et al*., 2006). SOCS2 has also been shown to enhance IL-2 and IL-3 signalling (Tannahill *et al*., 2005) by accelerating proteasome-dependent degradation of SOCS3. Similar effects again have been shown on signalling via the IFN type 1 and leptin receptors (Piessevaux *et al*., 2006). These observations imply that SOCS2 is counteracting the effects of other SOCS proteins, rather like a secondary negative feedback mechanism, to limit the effects of excessive levels of SOCS proteins. This assumption is supported by the findings that SOCS2 induction usually occurs a long time after cytokine stimulation and is prolonged, whereas SOCS1 and SOCS3 expression is typically quite rapid and transient (Adams *et al.*, 1998; Pezet *et al.*, 1999). Although quite poorly understood, SOCS6 and SOCS7 have also been shown to bind other SOCS members and similar effects to SOCS2 have been observed for SOCS6 (Piessevaux *et al*., 2006). Again, this data suggests that SOCS proteins can act as positive and negative regulators of signalling pathways and could explain some reported anomalies, such as the enhanced insulin signalling observed in transgenic mice overexpressing SOCS6 (Li *et al*., 2004) or the gigantism observed in transgenic mice overexpressing SOCS2 (Greenhalgh *et al*., 2002).

1.4.5.3 Functional Roles of SOCS Proteins

The functions of SOCS proteins have largely been elucidated by the generation of mice engineered to lack particular SOCS genes. These studies have greatly enhanced our understanding of the roles SOCS proteins play, particularly with regards to the immune response, and have also identified key definitive roles of individual SOCS members, such as the non-redundant role SOCS1 appears to play in IFNγ signaling (Alexander *et al.*, 1999; Marine *et al.*, 1999) (Table 3). However, this is not always the case and knock-out models can encounter problems. Due to placental insufficiency, SOCS3-null mice die at mid-gestation (Roberts *et al.*, 2001; Takahashi *et al.*, 2003). To overcome this, other ways of investigating SOCS3 deficiency have been explored. A genetic cross study conducted by Robb *et al*. (2005) showed that mice on a leukaemia-inhibitory factor (LIF)/SOCS3-null background were rescued from embryonic lethality due to placental failure, and the mice appeared normal at birth (Robb *et al*., 2005). It is believed that the deletion of SOCS3 leads to dyregulated LIF signalling through the LIFRα-chain, which alters trophoblast differentiation and causes placental defects (Boyle & Robb, 2008). In support of this is the finding that the number of trophoblast giant cells are reduced in LIFRα-null mice, compared with an abnormally high number of trophoblast giant cells in SOCS3-null mice (Takahashi *et al*., 2003). Although embryonic lethality is rescued, a high neonatal mortality rate is observed in $SOC3^{-/-}LIF^{-/-}$ mice and adult mice develop a fatal inflammatory disease, which is very similar to that seen in mice with a conditional deletion of SOCS3 in hematopoietic cells (Croker *et al*., 2004). $LIF^{-/-}$ mice, on the other hand have a normal lifespan and do not exhibit any major hematopoietic abnormalities. This suggests that SOCS3 plays a vital role in the negative regulation of the inflammatory response.

Another way to overcome SOCS3 embryonic lethality is the generation of conditional knock-outs, using the Cre recombinase and loxP system. In this way, the modified target gene can be ablated in adulthood, thus avoiding the placental insufficiency as observed with SOCS3 knock-outs. Furthermore, this ablation of the gene can be targeted to any tissue at any defined time. This is a powerful tool for the examination of genes that appear to be crucial during embryonic development, but may play important roles in particular adult tissues (Sauer,

1998). Applying this system with irradiated mice reconstituted with SOCS3$^{-/-}$ fetal liver cells, the SOCS3 gene has been specifically deleted in the liver and in macrophages. The absence of SOCS3 results in prolonged STAT3 and STAT1 activation following IL-6 treatment, but normal activation of STAT1 in response to IFNγ and normal activation of STAT3 in response to IL-10 (Croker *et al.*, 2003; Lang *et al.*, 2003). SOCS3 deficiency also upregulates several IFNγ-responsive genes following IL-6 treatment, which is not observed upon IL-6 stimulation of cells with functional SOCS3 alleles. This suggests that STAT1 provokes a dominant IFNγ-like gene expression profile owing to excessive STAT1 phosphorylation and activation. Furthermore, a mutation in gp130 (Tyr759Phe) in mice, which impedes SOCS3 and/or SHP2 recruitment, was shown to result in a phenotype displaying rheumatoid arthritis (RA)-like joint disease, a condition known to be associated with dysregulation of IL-6 signalling (Atsumi *et al*., 2002). Collectively, these studies show that SOCS3 is the main physiological regulator of IL-6 signalling and that SOCS3 can regulate the specificity of the cytokine response as well as the duration of the signal (Croker *et al.*, 2003; Lang *et al.*, 2003).

Interestingly, in the absence of SOCS3 in mouse macrophages, IL-6 has been shown to induce an 'IL-10-like' anti-inflammatory response, as demonstrated by a reduction in LPS-induced production of TNFα and IL-12, by IL-6 in SOCS3 deficient cells. (Yasukawa *et al*., 2003). This is interesting because there is currently no explanation as to why these two cytokines have such diverse effects. Both cytokines use identical JAK-STAT members and yet have very distinct gene expression patterns (Figure 1.12) (Murray, 2007). IL-10 has been shown to be anti-inflammatory in macrophages and dendritic cells, activating a different set of genes from IL-6, but both cytokines also activate a common pool of genes, including SOCS3 (Murray, 2007). Yasukawa *et al*. (2003) proposed that the difference in gene expression may be due to the intensity of the STAT3 signal. However, Murray (2006) has identified flaws in this concept, for example the strength of the signal does not account for the commonality of genes activated by the two cytokines. One obvious difference between the two cytokines is the involvement of SOCS3 as an inhibitory step in IL-6 signalling, but not in IL-10 signalling. Studies have shown that if modified receptors are used, which are either naturally insensitive to SOCS3 (e.g. IL-22R) or engineered to be insensitive

(e.g. IL-6, leptin receptors), but activate STAT3, an anti-inflammatory response is triggered (El Kasmi *et al*., 2006). Thus, based on SOCS3 involvement, a hypothesis has been proposed describing the activation of a generic pool of STAT3 by the IL-10R, which is not subjected to any inhibition by SOCS3. The IL-6R, on the other hand activates a different pool of STAT3, which is specifically inhibited by SOCS3, possibly *via* post-translational modification by kinases or phosphatases etc. These different pools of STAT3 may therefore go on to activate very different sets of genes. This is just one idea put forward by Murray (2007) and highlights the gaps in our understanding of the various modes of action of SOCS3 on signalling pathways and hence the importance of further studies.

With regards to leptin signaling, mice with a neural-specific deletion of SOCS3 have been generated using the Cre-loxP system. Similar to the IL-6 data presented above, SOCS3 deletion results in prolonged activation of STAT3 in response to leptin. Moreover, SOCS3 deficient mice exhibited a greater body weight loss when compared to their wild-type littermates. These knock-out mice were also resistant to high fat diet-induced weight gain and hyperleptinaemia, and retained insulin sensitivity. This study showed that SOCS3 is a key regulator of leptin signalling and hence plays an important role in diet-induced leptin and insulin resistance (Mori *et al*., 2004). A number of studies support this link between SOCS3 and leptin resistance, whereby leptin-mediated induction of SOCS3 has been associated with the attenuation of OB-Rb signalling (Bjorbaek *et al*., 1998). Chronic stimulation of OB-Rb has been shown to result in the desensitisation of OB-Rb signalling, whereby the receptor becomes refractory to re-stimulation. Mutation of the STAT3 binding site on OB-Rb (Tyr1138Ser), which mediates STAT3-induced SOCS3 induction, alleviates this feedback inhibition. Moreover, RNA interference-mediated knock-down of SHP2 had no effect on the attenuation of OB-Rb signalling, suggesting a role for SOCS3 in the feedback inhibition of OB-Rb signalling and not SHP2 (Dunn *et al*., 2005).

1.5 Aim

The anti-inflammatory effects of cAMP have been well documented, notably with regards to endothelial barrier function. However, the exact molecular mechanisms underlying these effects are still unclear. To investigate this further in the context of endothelial inflammation, the signalling pathways of IL-6 and leptin will be

examined in response to cAMP elevation in two endothelial cell types; human umbilical vein endothelial cells (HUVECs) and a novel human endothelial angiosarcoma-derived cell line (AS-M). Focus will be placed on SOCS3 as a potential mediator of the possible inhibitory effects, since previous experiments in the Palmer lab have demonstrated SOCS3 induction in response to cAMP elevation. Refer to Figure 1.13 for a diagram of the working hypothesis. In addition, this study also aims to further characterise AS-Ms in the context of both cytokine and cAMP signalling, since there are currently very limited studies on AS-Ms in the public domain. This endothelial cell line represents a less costly cell system when compared to HUVECs and could prove to be a tractable endothelial model for future research in endothelial biology.

Figure 1.1 The structure of AC

AC comprises 12 transmembrane (TM) domains, which can be divided into TM1 and TM2 and 2 cytoplasmic domains labelled C1 (in blue) and C2 (in red). The C1 and C2 domains can be further divided into C1a and C2a, which are highly conserved, forming the catalytic core and the less conserved C1b and C2b domains.

(Taken from Cooper *et al*., 2003)

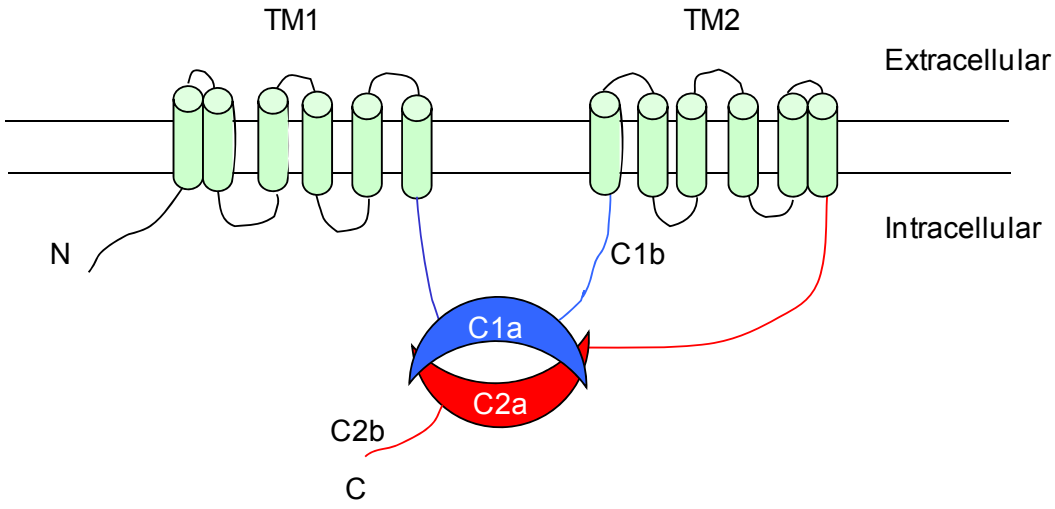


Figure 1.2 Schematic Representations of the Domain Structure of Epac1 and Epac2

CDC25HD – CDC25 homology domain with GEF activity, RA - Ras association domain, REM – Ras exchanger motif, B-site – cAMP binding domain, DEP – disheveled Egl-10 pleckstrin domain, A-site – cAMP binding domain.

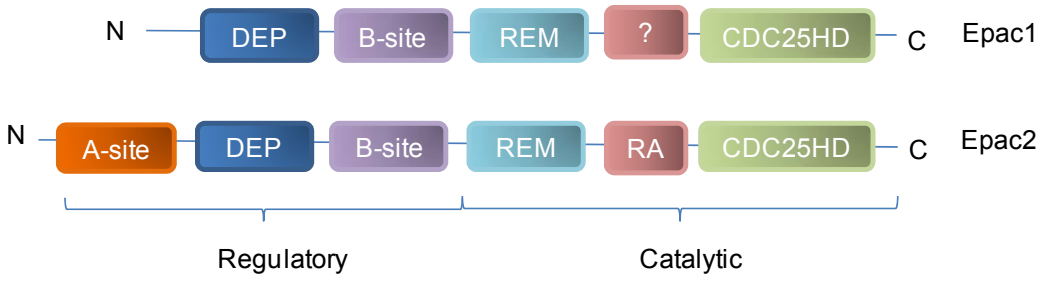


Figure 1.3 IL-6-type Receptor Complexes

All members of the IL-6-type cytokines signal *via* gp130, some *via* homodimers of gp130 like IL-6/IL-Rα, the majority *via* heterodimers of gp130 and LIFR, like LIF and lastly *via* heterodimers of gp130 and LIFR or OSM-R, like OSM

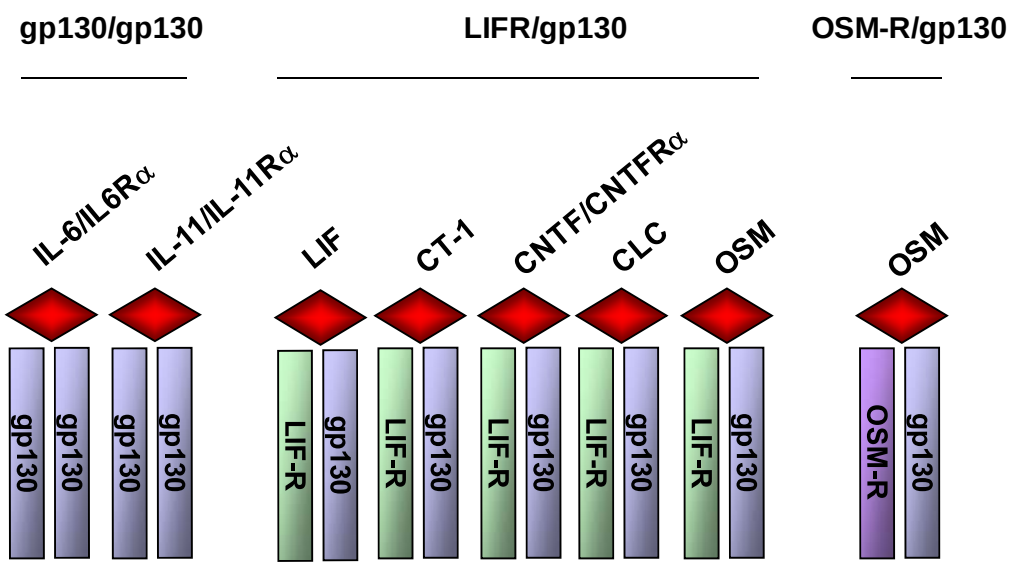


Figure 1.4 Structure of IL-6

IL-6 comprises 4 long α-helices, denoted A, B, C and D and receptor binding sites I, II and III shown by circles.
(Taken from (Heinrich *et al.*, 2003).

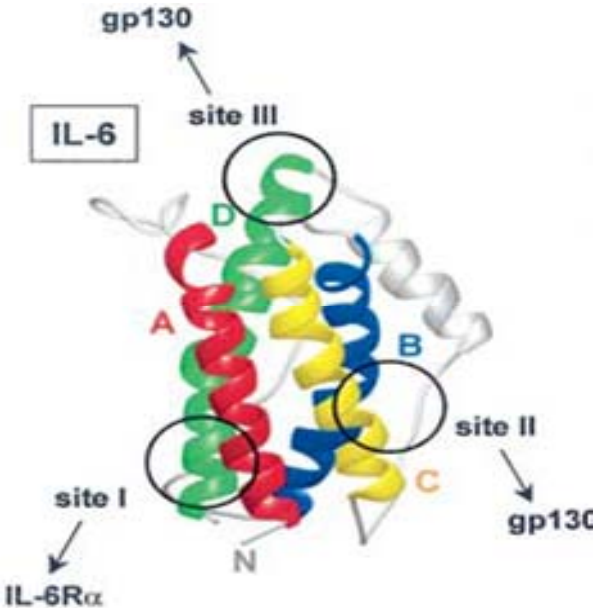


Figure 1.5a & b Schematic Representations of the JAK Homolgy (JH) and STAT Domains

The JH domanins are numbered 1-7, from the carboxyl to the amino terminus. These constitute the kinase, pseudo-kinase, SH2 and FERM domains. The STAT domains include the N-terminal dimerisation domain, the coiled-coil domain, the DNA binding domain, the linker domain, the SH2 domain and the C-terminal conserved tyrosine residues and transactivation domain.

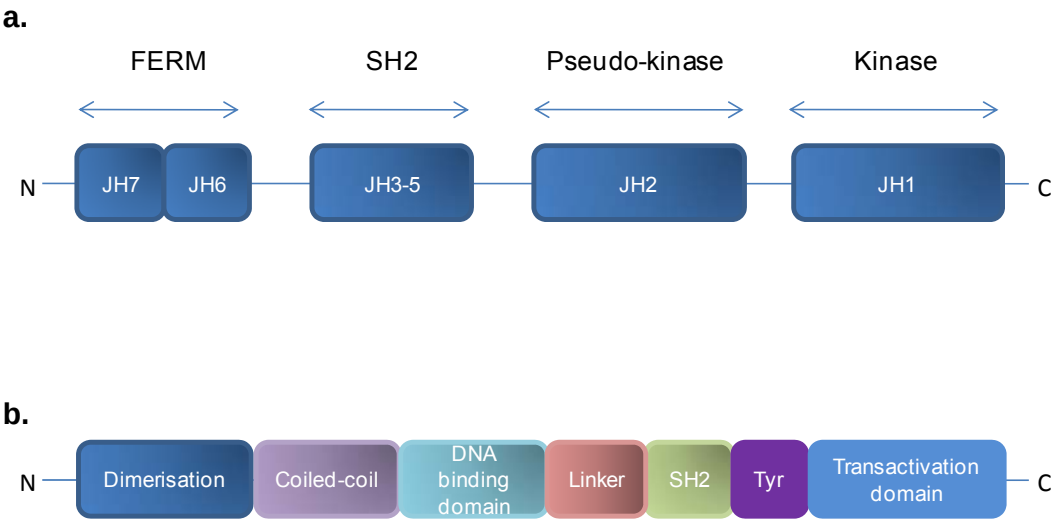


Figure 1.6 Evolutionary Conserved Raf-MEK-ERK Pathway

Conventionally, the serine/threonine kinase Raf (MAPKK), of which there are 3 isoforms; A-Raf, B-Raf and Raf-1, is activated by Ras GTPases, which are themselves activated by most cell-surface receptors. All 3 isoforms of Raf are capable of activating MEK (MAPKK) by phosphorylation of 2 serines in the activation loop. MEK is a dual-specificity kinase, which activats ERK (MAPK) by phosphorylating threonine and tyrosine residues in a TEY motif, present in the activation loop. Both MEK and ERK have two isoforms, which are mostly co-regulated.

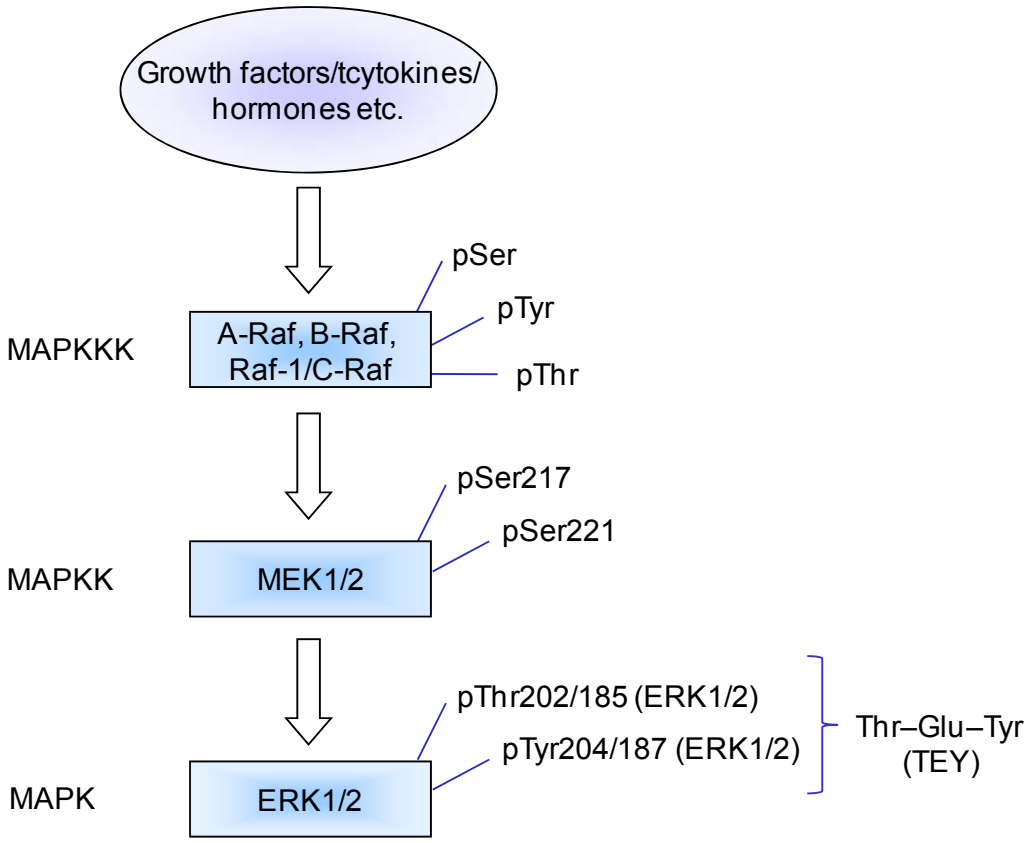


Table 1 ERK 1,2 Substrates

More than 150 ERK1/2 substrates have been identified. These substrates can be classified into transcription factors, protein kinases and phosphatases, cytoskeletal proteins, signalling proteins, apoptotic proteins and proteinases, and other types of proteins. (Table taken from (Lu & Xu, 2006).

Transcription factors	Kinases and phosphatases	Cytoskeletal proteins	Signalling proteins	Apoptotic proteins & proteinases	Other proteins
AML1 (RUNX1)	DAPK	Annexin XI	EGFR	Bad	Amphiphysin 1
Androgen receptor	ERK1/2	Caldesmon	ENaCβ/γ	Bim-EL	CPSII/CAD
ATF2	FAK1	Calnexin	Fe65	Calpain	CR16
BCL6	GRK2	CENP-E	FRS2	Caspase 9	GRASP55
BMAL1	Inhibitor-2	Connexin-43	Gab1	EDD	GRASP65
CBP	Lck	Cortactin	Gab2	IEX1	HABP1
C/EBPβ	MAPKAP3	Crystallin	GAIP	MCL-1	Histone H
CRY1/2	MAPKAP5	DOC1R	Grb10	TIS2	HnRNP-K
E47	MEK1/2	Dystrophin	IRS1	TNFR CD120a	KIP
Elk1	MKP1/2	Lamin B2	LAT		MBP
ER81	MKP7	MAP1	LIFR		PHAS-I
ERF	MLCK	MAP2	MARCKS		CPLA2
Estrogen receptor	MNK1/2	MAP4	Naf1α		Rb
c-Fos	MSK1/2	MISS	PDE4		SAP90/PSD95
Fra1	PAK1	NF-H	PLCβ		Spinophilin
GATA1/2	PTP2C	NF-M	PLCγ		Topoisomerase II
HIF1α	Raf1	Paxillin	Potassium channel Kv 4.2		Tpr
HSF1	B-Raf	Stathmin	KSR1		TTP (Nup47)
ICER	RSK1-4	SW1/SNF	Rab4		Tyrosine hydroxylase
c-Jun	S6K	Synapsin 1	SH2-B		Vif
Microphthalmia	Syk	Tau	ShcA		Vpx
c-Myc		Vinexin β	Sos1		
N-Myc			Spin90		
Net (Sap2)			TSC2		
NFATc4					
NF-IL6					
NGFI-B/TR3/Nur77					
Pax6					
PPARγ					
P53					
Progesterone receptor					
RNA Pol. II					
PUNX2					
Sap1					
Smad1					
Smad2/3					
SP1					
SRC1					
SREBP1/2					
STAT1/3					
STAT5a					
TALI1/SCL					
TFII-I					
TFIIIB					
TGIF					
TIF1A					
UBF					

Figure 1.7 IL-6-activated STAT and ERK1,2 Signalling Cascade

IL-6 signals *via* gp130 homodimers. IL-6 cannot bind and activate gp130 directly, it first needs to bind an IL-6 receptor α-subunit (IL-6Rα). ECs do not express IL-6Rα, but soluble forms of the receptor, termed soluble IL-6Rα (sIL-6Rα) are shed from adjacent leukocytes at sites of inflammation. The sIL-6Rα/IL-6 *trans-signalling* complex binds and activates gp130, which causes conformational changes in the gp130 homodimer. JAK1, which is bound to gp130 *via* conserved box1 and box2 regions, becomes phosphorylated as a result of these conformational changes. JAK1 phosphorylation and activation leads to the phosphorylation of specific Tyr residues, which provide docking sites for SH2 domain-containing proteins for downstream signalling. Two major pathways activated by gp130 are the STAT pathway and the ERK pathway. PTyr759 serves as a docking site for SHP2, which becomes phosphorylated *via* JAK1 and leads to the activation of the ERK pathway. PTyr767, 814, 905 and 915 are all docking sites for STAT3, whereas pTyr905 and 915 can also recruit STAT1. STAT3 appears to be the predominant STAT activated during IL-6 signalling. Following STAT phosphorylation *via* JAK1, homo-and/or heterodimerisation complexes are formed.

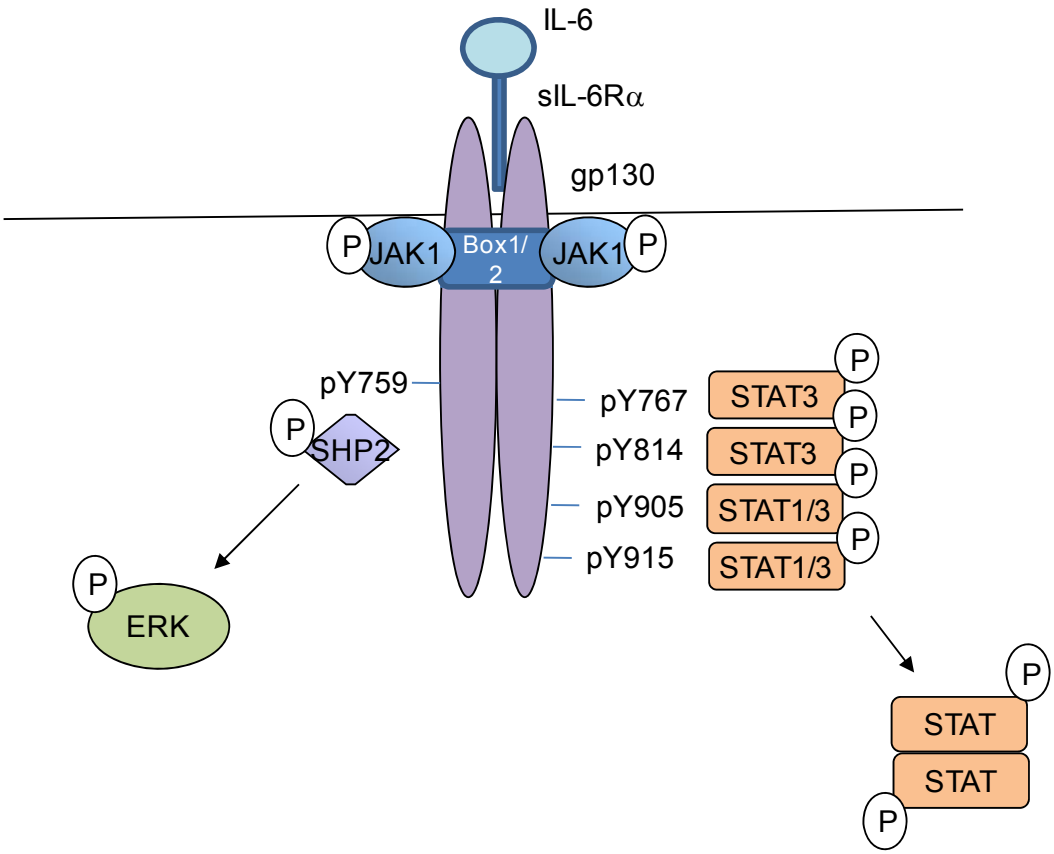

IL-6
sIL-6Rα
gp130
P
JAK1
Box1/2
JAK1
P
pY759
P
SHP2
pY767
pY814
pY905
pY915
P
STAT3
P
STAT3
P
STAT1/3
P
STAT1/3
P
ERK
P
STAT
STAT
P

Figure 1.8 Leptin Receptors

A diagram of the 6 leptin isoforms, OB-Ra - f, showing the cytokine homology domains (CHD_2), the fibronectin type III domains (F-III), the box1, 2 motifs and the phospho-Tyr residues of OB-Rb.

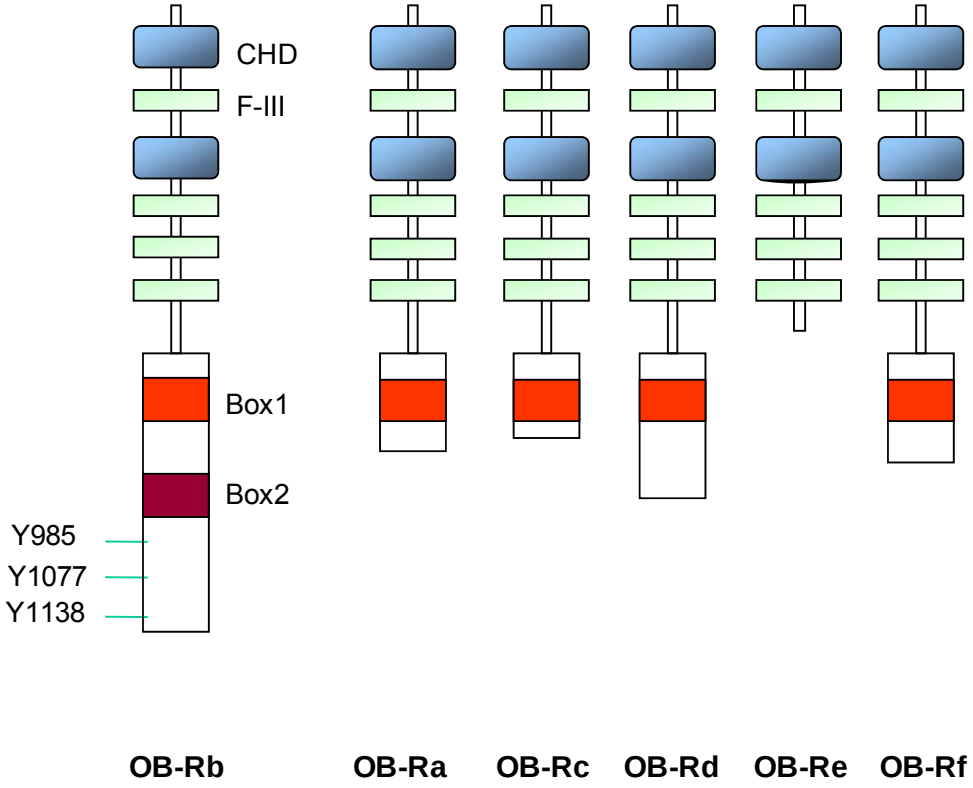


Figure 1.9 Leptin-activated STAT and ERK1,2 Signalling Cascade

Leptin signals *via* OB-R homodimers. Leptin can bind to one of six isoforms of OB-R, however, only OB-Rb has full signalling capability, due to the presence of key phosphoTyr residues on its intracellular domain (Y985, Y1077 and Y1138). Leptin binding causes conformational changes in the OB-R homodimer, which allow JAK2 transphosphorylation. JAK2 is associated with the conserved box1 region on OB-R. JAK2 phosphorylation and activation leads to the phosphorylation of specific Tyr residues, which provide docking sites for SH2 domain-containing proteins for downstream signalling. Two major pathways activated by OB-Rb are the STAT pathway and the ERK pathway. PTyr985 serves as a docking site for SHP2, which becomes phosphorylated *via* JAK2 and leads to the activation of the ERK pathway. In addition, ERK activation has also been demonstrated in the absence of OB-Rb phosphorylation, yet requires JAK2 activation. With regards to STAT activation, pTyr1077 provides a docking site for STAT5, whereas pTyr1138 recruits STAT1, STAT3 and STAT5. However, STAT3 appears to be the predominant STAT activated during leptin signalling. Following STAT phosphorylation *via* JAK2, homo-and/or heterodimerisation complexes are formed.

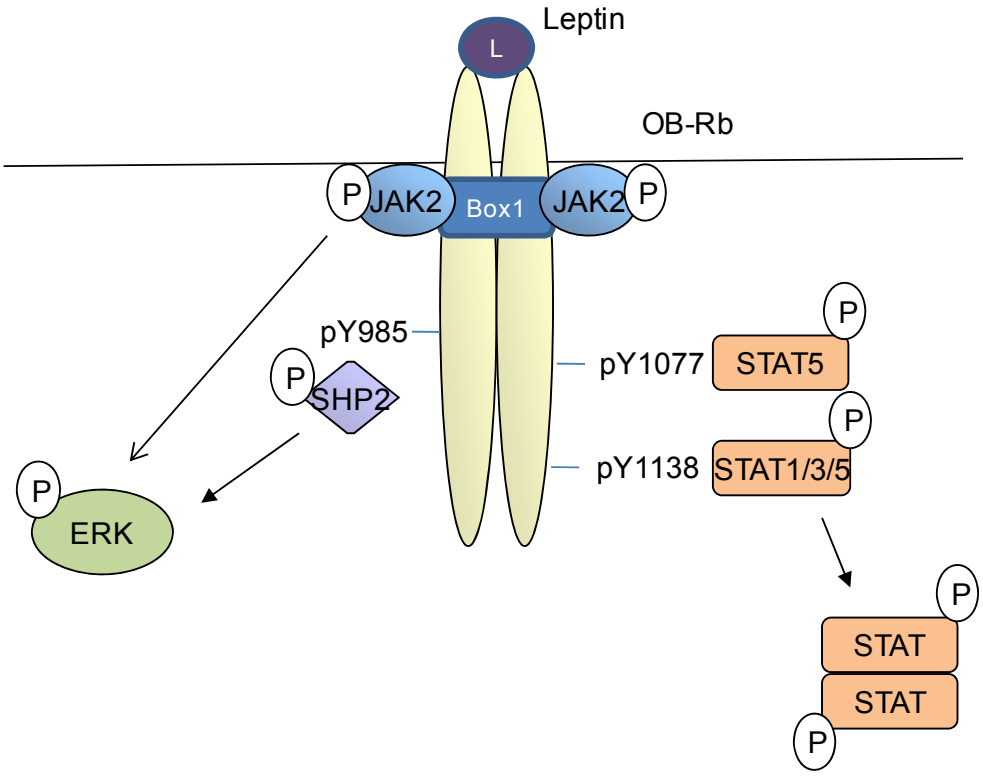

Leptin
L
OB-Rb
P
JAK2
Box1
JAK2
P
pY985
P
SHP2
pY1077
STAT5
P
P
pY1138
STAT1/3/5
P
ERK
P
STAT
STAT
P

Table 2 PIAS Regulated Proteins

PIAS proteins have been shown to regulate more than 60 proteins, with most of these constituting transcription factors. The proteins in the table are the main proteins shown to interact with PIAS proteins in immune regulation.
(Taken from Shuai & Liu, 2005)

Interacting Protein	PIAS
Inteferon Pathways	
STAT1	PIAS1, PIASγ, PIAS3, PIASx-α
STAT3	PIAS3
STAT4	PIASx
STAT5	PIAS3
GFI1	PIAS3
ATBF1	PIAS3
IRF1	PIAS3
IRF3, -7	PIASγ
NF - κB Pathways	
NF - κB p65	PIAS1, PIAS3
TRIF	PIASγ
SMAD Pathways	
SMAD3	PIAS3, PIASγ
SMAD4	PIAS1, PIASx-β
Oncoproteins and Tumour Suppressor Proteins	
p53	PIAS1, PIASx-β
p73	PIAS1, PIASx
JUN	PIAS1, PIASx
MYB	PIASγ
MDM2	PIAS1, PIASx-β
PLAG1	PIAS1, PIASγ
LEF1	PIASγ
Viral Proteins	
IE2	PIAS1
RTA	PIAS1
E1	PIAS1, PIASx
NP	PIAS1, PIASx-β
Others	
C/EBP-ε	PIAS1, PIASx
SATB2	PIAS1
MITF	PIAS3

Figure 1.10 Schematic Representation of the Model of SHP2 Phosphatase Activation

In the inactive state, the SH2 domain of SHP2 appears to sterically hinder the access of phosphoTyr substrates to the protein Tyr phosphatase (PTP) domain and inhibit phosphatase activity. Activation of the phosphatase occurs in 2 ways; (1) the SH2 domains can bind to phosphoTyr motifs on activated receptors, which leads to unfolding of the protein and subsequent phosphatase activation or (2) the SH2 domains can bind to the phosphoTyr residues 542 and 580 on the C-terminal tail of SHP2 itself and cause conformational changes, which leads to activation.

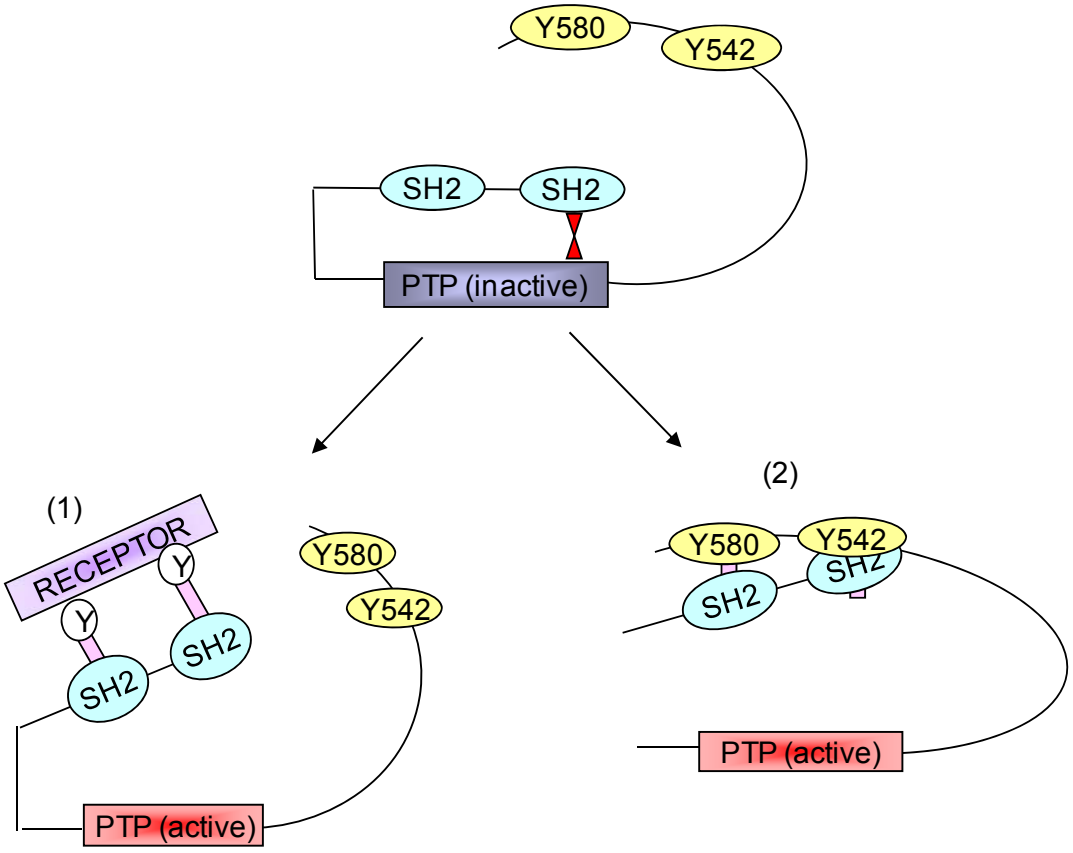


Figure 1.11 The Three Main Mechanisms Utilised by SOCS Proteins to Downregulate Cytokine Signalling

(1) Binding of SOCS proteins to specific phosphotyrosine residues on receptors and physically blocking occupation of docking sites of other SH2 domain-containing signalling proteins, such as SHP2.

(2) The kinase inhibitory region (KIR) of SOCS1 and SOCS3 binding to the kinase domain of JAK and inhibiting JAK activation.

(3) The SOCS box of SOCS proteins recruiting the ubiquitin-ligase machinery and promoting the proteosomal degradation of signalling proteins bound to SOCS via their SH2 domains.

The orange oval represents SH2 domain-containing signalling proteins.

(Adapted from Howard & Flier, 2006)

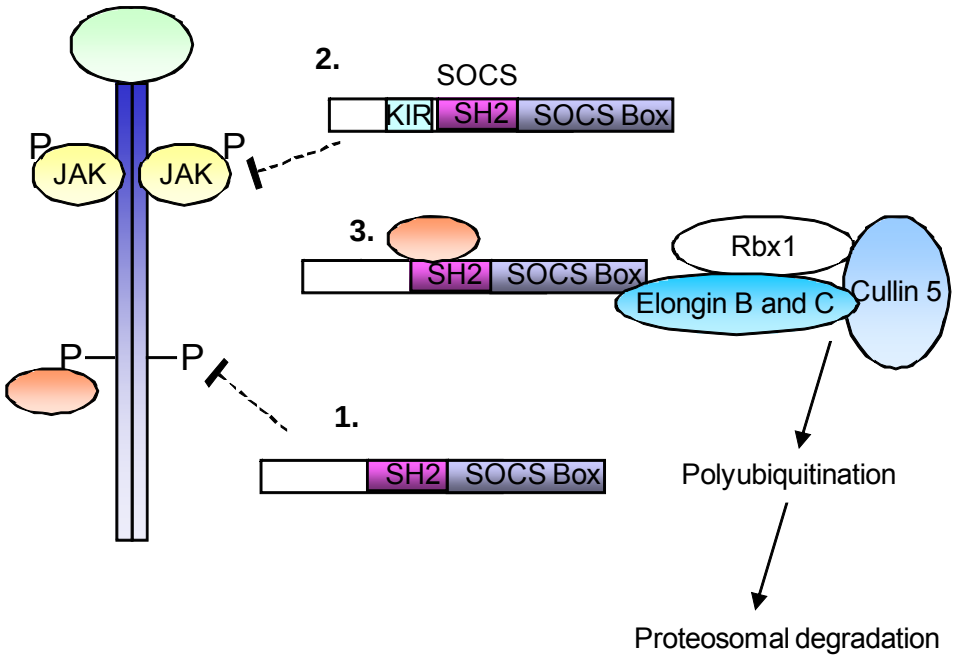


Table 3 SOCS Knockout in Mice

The major consequences of SOCS knockout *in vivo*, with tissue-specific knock-outs described in the text (Section 1.4.5.3).

Gene	**Knockout mice**
CIS	Has no detectable abnormal phenotype (Marine *et al*. 1999).
SOCS1	Neonatal lethality, due to dysregulation of IFNγ signalling. (Marine *et al*., 1999). Additionally, fatty degeneration in liver, hematopoietic infiltration of multiple organs and lymphoid deficiencies (Starr *et al.*, 1998).
SOCS2	Gigantism and dysregulation of GH signalling (Metcalf *et al.*, 2000).
SOCS3	Embryonic lethality due to placental defects (Marine *et al*., 1999, Roberts *et al*., 2001, Takahashi *et al*., 2003).
SOCS6	Mild growth retardation (Krebs *et al.*, 2002).
SOCS7	Enhanced insulin sensitivity and increased growth of pancreatic islets (Banks *et al.*, 2005).

Figure 1.12 IL-6 and IL-10 Signalling

IL-6 and IL-10 signal *via* identical JAK-STAT members, but activate different sets of genes; IL-10 activates a subset of anti-inflammatory genes, whereas IL-6 activates a sub-set of IL-6 regulated genes. SOCS3 is representative of a 'common' subset of genes activated by both cytokines.
(Taken from Murray *et al*., 2007)

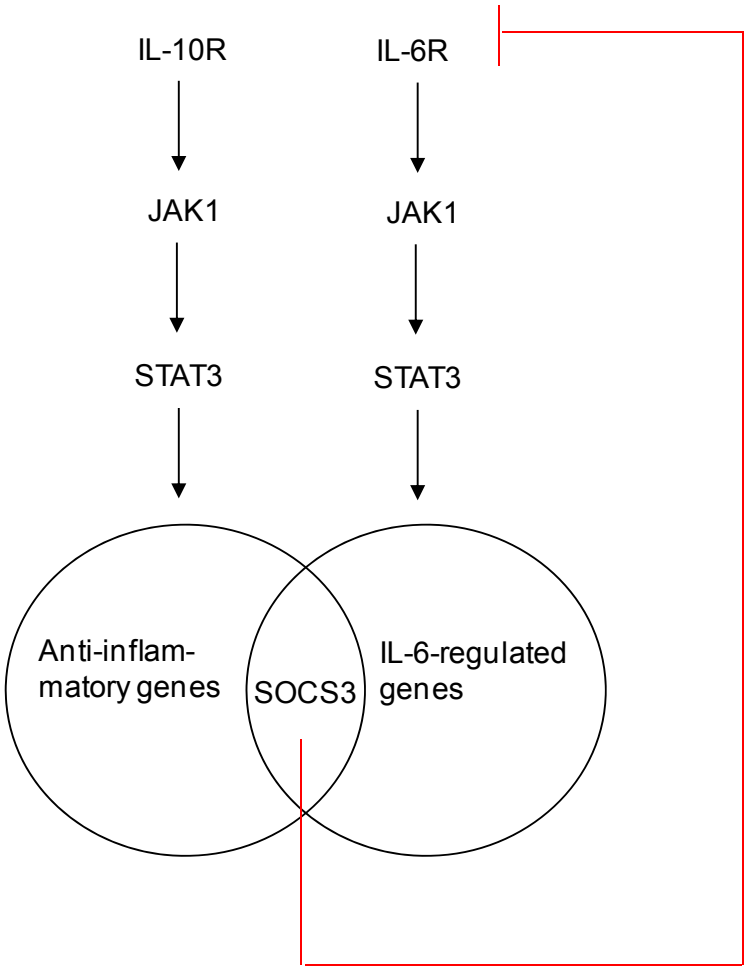
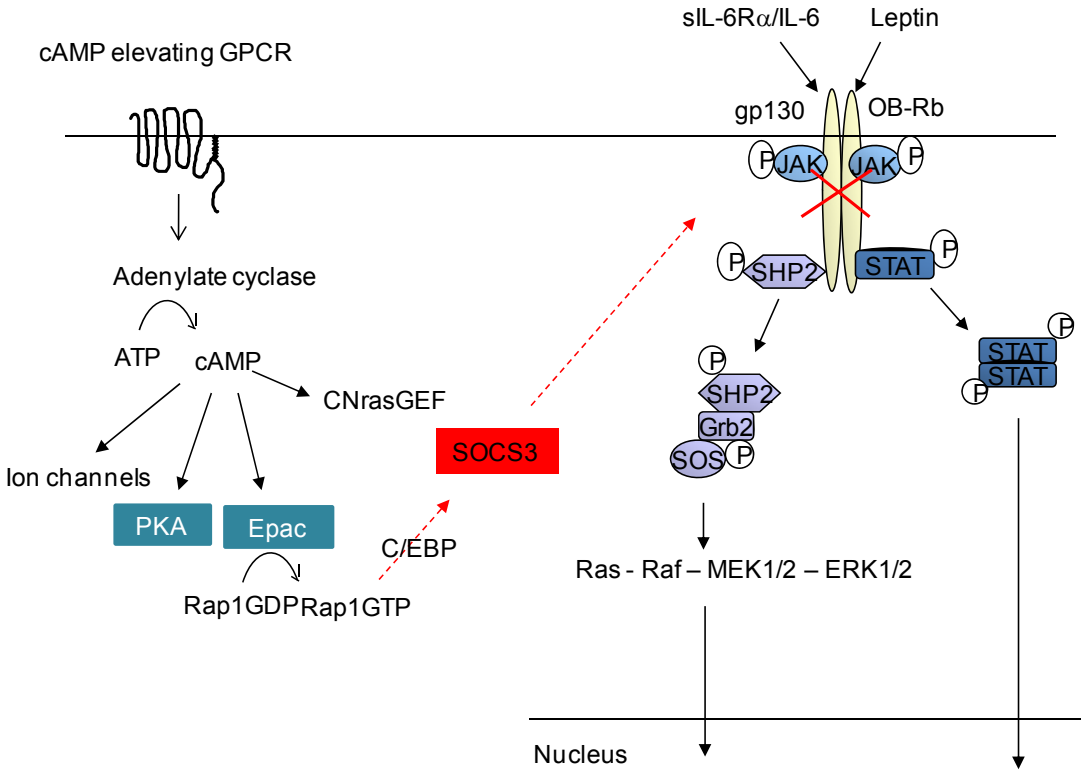


Figure 1.13 Diagram of the Working Hypothesis

cAMP-elevating stimuli, such as β-adrenergic catecholamines, histamine, prostaglandins and adenosine bind to their respective G protein coupled receptors (GPCRs) to activate the adenylyl cyclase signalling pathway, resulting in increased intracellular cAMP levels. cAMP then activates Epac, a cAMP-activated guanine nucleotide exchange factor for the small GTPases Rap1 and Rap2. Active Rap1GTP goes on to trigger SOCS3 induction, possibly *via* the C/EBP family of transcription factors. SOCS3 accumulation subsequently leads to the inhibition of sIL-6Rα/IL-6-and leptin-stimulated ERK1,2 and STAT3 activation.



Chapter 2

Materials and Methods

2.1 Materials

Abcam plc., Cambridge, UK

SOCS3 Ab	(cat. no. ab16030)
Phospho-CREB (Ser133) Ab	(cat. no. ab3419)
Sp1 Ab	(cat. no. ab13370)

Autogen Bioclear UK Ltd., Calne, UK

SOCS3 si-RNA (h)	(cat. no. sc-41000)
Control siRNA-A	(cat. no. sc-37007)

Biolog Life Science Institute, Bremen, Germany

8-(4-Chlorophenylthio)-2'-O-methyl-cAMP	(cat. no. C 041)

Cell Signaling Technology (New England BioLabs UK Ltd.) Herts, UK

Phospho-p44/42 MAPK (Thr202/Tyr204) mouse mAb	(cat. no. 9106)
Phospho-STAT3 (Tyr705) mouse mAb	(cat. no. 9138)
p44/42 MAPK Ab	(cat. no. 9102)
STAT3 Ab	(cat. no. 9132)

Dharmacon RNA Technologies, Lafayette, CO

Epac1-targeted siRNA	(cat. No. M-007676-00)

GE Healthcare UK Ltd., Buckinghamshire, UK

Rainbow molecular weight marker	(cat. no. RPN 756)

Inverclyde Biologicals, Bellshill, UK

Protan nitrocellulose transfer membrane	(cat. no. 10401396)

Invitrogen Ltd., Paisley, UK

Opti-MEM®, reduced serum medium	(cat. no. 31985-047)
Oligofectamine reagent	(cat. no. 12252-011)

Lonza Wokingham Ltd., Berkshire, UK

Dulbecco's Phosphate Buffered Saline (DPBS)	(cat. no. BE17-512F)
EGM bulletkit	(cat. no. CC-3162)
Pooled HUVEC cryopreserved in EGM	(cat. no. CC-2519)

Merck Biosciences, Nottingham, UK

MG-132	(cat. no. 474790)
Forskolin	(cat. no. 344270)
Rolipram	(cat. no. 557330)
H89	(cat. no. 371963)
U0126	(cat. no. 662005)
Phorbol-12-myristate-13-acetate (PMA)	(cat. no. 524400)

Perkin Elmer Life and Analytical Sciences, Monza, Italy

Enhanced chemiluminescence (ECL) reagents	(cat. no. NEL 104)

Pierce (Perbio Science UK Ltd.) Northumberland, UK

Restore western blot stripping buffer	(cat. no. 21059)

Promega, Southampton, UK

Wizard plus miniprep DNA purification system	(cat. no. A7100)
Passive lysis 5x buffer	(cat. no. E1941)

Qiagen Ltd., Crawley, UK

Endofree plasmid maxi kit	(cat. no. 12362)

R & D Systems Europe Ltd., Abingdon, UK

Recombinant human leptin (OB)	(cat. no. 398-LP)
Recombinant human IL-6	(cat. no. 206-IL)
Recombinant human sIL-6Rα	(cat. no. 227-SR)

Roche Diagnostics Ltd., Burgess Hill, UK

Fugene® 6 transfection reagent	(cat. no. 11815091001)
Agarose	(cat. no. 1388991)

Santa Cruz Biotechnology Inc., Heidelberg, Germany

SOCS3 (m-20) goat polyclonal Ab	(cat. no. sc-7009)
SOCS3 siRNA (h)	(cat. no. sc-41000)
Control siRNA-A	(cat. no. sc-37007)

Sigma-RBI, Dorset, UK

Anti-α-tubulin mAb	(cat. no. T9026)
Sterile filtered cell culture water	(cat. no. W3500)
Trypsin – EDTA	(cat. no. T4299)
Tween – 20	(cat. no. P5927)
30% (w/v) acrylamide/0.8% (w/v) bis-acrylamide	(cat. no. A3699)
L-glutamine	(cat. no. G7513)
Penicillin-streptomycin	(cat. no. P0781)
Anti-mouse IgG (peroxidase-conjugated)	(cat. no. A4416)
Anti-rabbit IgG (peroxidase-conjugated)	(cat. no. A6154)
Soybean trypsin inhibitor	(cat. no. T9003)
Benzamidine	(cat. no.12072)
Bovine serum albumin	(cat. no. A7030)
Bromophenol blue	(cat. no. B7021)
Ampicillin	(cat. no. A9393)
N, N, N',N'-tetramethylethylenediamine (TEMED)	(cat. no. T9281)
Phenylmethylsulphonyl fluoride (PMSF)	(cat. no. P7626)
Medium 199	(cat. no. M7528)
Nonidet P-40	(cat. no. N6507)
Tissue culture bovine serum albumin	(cat. no. A1595)

Wild-type ($SOCS3^{+/+}$) and $SOCS3^{-/-}$ murine embryonic fibroblasts (MEFs) (Kawaguchi *et al*., 2004) were generously provided by Prof. Akihiko Yoshimura (Kyushu University, Japan).

Monoclonal anti-Epac1 antibody 5D3 (Price *et al*., 2004) was generously supplied by Prof. Johannes Bos (University Medical Center, Utrecht, Netherlands).

2.2 Methods

2.2.1 Cell Culture

HUVECs and AS-Ms were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 in EGM-2 supplemented with 2% (v/v) foetal bovine serum (FBS), plus hydrocortisone, hFGF, VEGF, R^3-IGF, ascorbic acid, hEGF, GA-1000 (Gentamicin, Amphotericin-B) and heparin, as recommended by the supplier (Lonza), which will hereafter be referred to as EGM. MEFs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) FBS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin, which will hereafter be referred to as DMEM. Cells were grown until 70% confluence before being passaged and seeded into plates or dishes for experimentation or flasks for further propagation. Passaging the cells involved washing the cells once with tissue grade PBS and adding 2ml of endothelial trypsin (HUVECs and AS-Ms) and 2ml of trypsin (MEFs) to each flask. Cells were left for a few minutes at 37°C to allow detachment from the flask. Fresh DMEM was added to the MEF cell mixture and gently pipetted to resuspend the cells. The HUVEC/AS-M cell mixture was added to a 50ml tube containing EGM to neutralise the trypsin and spun down at 200 g for 5 minutes. The cell pellet was then resuspended in fresh medium. HUVECs and AS-Ms were counted using a haemocytometer and seeded at the following densities; 3-4x10^5 cells per well of a 6 well plate, 12x10^5 cells per 10cm^2 dish.

2.2.2 Transfection of cells using Oligofectamine

Based on 1 well of a 6 well plate, 3µl Oligofectamine was added to 12µl Optimem and incubated for 10 minutes at room temperature. Oligonucleotides were resuspended to give the relevant stock concentrations (10µM SOCS3 siRNA, 20µM Epac1 siRNA). The appropriate volumes of these oligonucleotides were then used for experimentation (based on prior optimisation experiments) and made up to a final volume of 185µl with Optimem. Following 10 minutes, the Oligofectamine mix was added to the oligonucleotides and incubated for a further 20 minutes at room temperature. During this time, the cells were washed twice with Optimem and 1ml Optimem per well was added to the cells. 200µl of transfection mix per well was added dropwise to the cells, which were then placed back in the incubator for 5 hours. Following 5 hours, 1ml EGM was added to each

well and the cells left overnight at 37°C. This procedure was repeated the next day and the experimental conditions were applied on the third day.

2.2.3 Transfection of cells using Fugene

Based on 1 well of a 6 well plate, 0.5-1μg of endotoxin-free DNA was added to 200μl serum-free medium (Optimem) and mixed. 10μl Fugene was added to the DNA suspension and mixed again. The transfection mix was then incubated for 15 minutes at room temperature before being added dropwise over the cells of each well. The cells were incubated overnight at 37°C and the following day the medium was replaced with fresh EGM.

2.2.4 Preparation of Cell Extracts for Immunoblotting

Following treatment of cells with the relevant stimuli, cells were transferred to ice. The medium was removed and cells were washed twice with ice cold PBS. Cells were then scraped down into 50μl RIPA lysis buffer (50mM HEPES pH7.5, 150mM NaCl, 1% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.01M sodium phosphate, 5mM EDTA, 0.1mM PMSF, 1μg/ml soybean trypsin inhibitor, 1μg/ml benzamidine) and transferred to ice cold micro-centrifuge tubes. The tubes were incubated on ice for 20 minutes to allow the proteins to solubilise before centrifugation at 20,000 g for 5 minutes at 4°C. The supernatant was transferred to fresh micro-centrifuge tubes for determination of protein concentration and analysis by SDS-PAGE and immunoblotting.

2.2.5 Bicinchoninic acid (BCA) Assay to Determine Protein Concentration

Bovine serum albumin (BSA) standards, ranging from concentrations of 0-2 mg/ml were added in duplicate to a 96 well plate in volumes of 10μl per well. The same volume of each cell lysate sample of unknown concentration was added to the plate in duplicate. 200μl of BCA solution (1% (w/v) 4, 4 dicarboxy-2, 2 biquinoline disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium bicarbonate pH 11.25, 0.08% (w/v) copper (II) sulphate) was added to each well and incubated at room temperature for 10 minutes. Following incubation, protein concentration was determined by measuring the absorbance of samples at 492nm (A_{492}) using a

MRX-TCII plate reader (Dynex Technologies). The absorbance values of the standards were used to construct a best-fit straight-line plot, from which the values of the unknown protein samples could be determined.

2.2.6 Bradford Assay to Determine Protein Concentration

Bovine serum albumin (BSA) standards, ranging from concentrations of 0-2 mg/ml were added in duplicate to a 96 well plate in volumes of 10μl per well. The same volume of each cell lysate sample of unknown concentration was added to the plate in duplicate. 50μl of Bradford's reagent, which had previously been diluted 1:4 with distilled deionised water, was added to each well and the samples incubated at room temperature for 5 minutes. Following incubation, protein concentration was determined by measuring the absorbance of samples at 630nm (A_{630}) using a MRX-TCII plate reader (Dynex Technologies). The absorbance values of the standards were used to construct a best-fit straight-line plot, from which the values of the unknown protein samples could be determined.

2.2.7 SDS-PAGE and Immunoblotting

Each sample was equalised for protein concentration and volume, and 2 x SDS-PAGE sample buffer (50mM Tris pH 6.8 at room temperature, 10% (v/v) glycerol, 12% (w/v) SDS, 10mM dithiothreitol, 0.0001% (w/v) bromophenol blue) was added. Samples were then subjected to fractionation by SDS-PAGE using a 10% or 12% acrylamide resolving gel (10%/12% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH8.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and a 3% acrylamide stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH6.8), 0.1% (w/v) SDS, 0.001% (w/v) ammonium persulphate and 0.001% (v/v) TEMED). Electrophoresis of samples was performed in the presence of pre-stained protein makers (Rainbow Markers 14.3 – 220kDa) using Biorad Mini-Protean III gel electrophoresis systems and running buffer (24.7mM Tris, 0.19M glycine, 0.1% (w/v) SDS) at 150V. The electrophoresis of samples and markers was stopped when the bromophenol blue dye front reached the bottom of the gel. Fractionated proteins were then electrophoretically transferred to a nitrocellulose membrane at 400mA for 45 minutes in transfer buffer (24.7mM Tris, 0.19M glycine, and 20%

(v/v) methanol). Following transfer the membranes were washed in Tris Buffered Saline-Tween (TBST) (20mM Tris, pH7.5, 150mM NaCl, 0.1% (v/v) Tween 20) once and transferred to blocking solution (5% (w/v) skimmed milk in TBST) for a 1 hour incubation with shaking at room temperature. Membranes were then incubated with primary antibody diluted in 5% BSA (w/v) in TBST overnight at 4°C on a rotating platform. The next day, the membranes were washed three times (10 minutes/wash) in TBST and incubated with the appropriate HRP-conjugated secondary antibody diluted in blocking solution (5% (w/v) skimmed milk in TBST) for 1 hour on a rotating platform at room temperature. The membranes were washed a further three times in TBST and finally incubated with ECL reagents as per the manufacturer's instructions. Immunoreactive proteins were visualised using X-ray film and the optical density of the bands was measured using the TotalLab 2003.02 software. Briefly, TotalLab analysis involved a step-wise manual approach to allow lane creation (using a semi-automated approach to define the number of lanes), background substraction (using the Rolling Ball method) and band detection (using 3 parameters; minimum slope, noise reduction and percentage maximum peak). Normalisation was performed as appropriate for each set of data and the values expressed as a proportion of the selected band.

2.2.8 Preparation of Antibiotic Agar Plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 1.5% (w/v) agar), which had been autoclaved and left to cool before addition of 50μg/ml ampicillin was poured into 10cm Petri plates. These plates were left overnight at room temperature to solidify. The next day, plates were transferred to 4°C for storage.

2.2.9 Plasmid DNA Preparation

A stab from a glycerol stock was inoculated with 10ml of LB broth (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride), supplemented with 50μg/ml ampicillin. This was then placed in a shaking incubator overnight at 37°C. The next day, plasmid DNA was extracted from the culture by using the Promega™ Wizard Plus SV mimiprep purification kit as per the manufacturer's instructions. Alternatively, the culture was transferred to 250ml of LB broth,

supplemented with 50μg/ml ampicillin and placed back in the shaking incubator overnight at 37°C. The next day, plasmid DNA was extracted from the culture using the Qiagen endofree plasmid maxi kit as per the manufacturer's instructions.

2.2.10 Transformation of Competent XL1 Blue *E.coli* cells

One vial of XL1 Blue cells was defrosted on ice, before transferring 80μl to a pre-chilled micro-centrifuge tube. Approximately 30-40ng of plasmid DNA was added to the tube and left on ice for 20 minutes. The tube was then incubated at 42°C for 2 minutes and placed back on ice. 1ml of LB broth was added to the tube and incubated for a further 30 minutes at 37°C. Following incubation, 100μl of the transformation mix was plated out onto agar plates containing 50μg/ml ampicillin and incubated overnight at 37°C.

2.2.11 Reporter Gene Assay

HUVECs and AS-Ms were seeded into 6 well plates at densities of 3 x 10^5 cells/well and 3.5 x 10^5 cells/well respectively. Cells were left for 24 hours prior to replacing the medium with fresh EGM. 1 hour after the medium change, cells were transfected with a *trans*-activator plasmid (Gal4-Elk-1) at 1μg/well, a Firefly luciferase reporter plasmid (Gal4-luc) at 1μg/well and a normalisation *Renilla* luciferase plasmid (pRL-CMV) at 0.5μg/well using Fugene reagent as described in section 2.2.3. The cells were then incubated overnight at 37°C, prior to another medium change with fresh EGM. The cells were left for a further 24 hours before treatment with or without leptin (125ng/ml), sIL-6Rα/IL-6 (25ng/ml/5ng/ml) or PMA (1μM) for 6 hours. Following treatment, cells were lysed in 50μl passive lysis buffer and stored at -80°C. Firefly and *Renilla* luciferase buffers were prepared fresh for each experiment; Firefly luciferase buffer pH8 (25mM Gly-gly, 15mM K_XPO_4 pH8, 4mM EGTA, 2mM ATP, 1mM DTT, 15mM $MgSO_4$, 0.1mM CoA enzyme and 0.075mM luciferin), *Renilla* luciferase buffer pH5 (1100mM NaCl, 2.2mM Na_2EDTA, 220mM K_XPO_4 pH5, 0.44mg/ml BSA, 1.3mM NaN_3 and 0.0143mM coelentrazine). 30μl of each cell lysate was transferred to a white-walled 96 well plate. The plate was then placed into the Luminoskan Acsent luminometer and 100μl of each of the luciferase buffers was automatically added to each well and luminescence was measured.

2.2.12 Nuclear Extraction

$12x10^5$ HUVECs or AS-Ms were seeded into $10cm^2$ dishes. 24 hrs after seeding, cells were washed twice in PBS and serum starved for 4 hrs. Following 4 hrs, cells were treated with or without leptin (125ng/ml) or sIL-6Rα/IL-6 (25ng/ml/5ng/ml) for 30 minutes or PMA (1μM) for 5 minutes. Cells were transferred onto ice and washed twice with cold PBS before adding 1ml PBS to each dish. The cells were scraped down and transferred to ice cold micro-centrifuge tubes for centrifugation at 5000 g for 5 minutes at 4°C. The cell pellet was resuspended in 400μl of ice cold buffer A (10mM HEPES (pH 7.9), 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT), supplemented with protease inhibitors on the day (0.1mM PMSF, 10μg/μl soybean trypsin inhibitor and 10μg/μl benzamidine) and left on ice for 10 minutes to allow the cells to "swell". 25μl of 10% (v/v) NP40 was then added to each sample to lyse the cells. Each sample was centrifuged again at 13,000 g at 4°C for a few seconds to pellet the nuclear fraction. The supernatant containing the cytosolic fraction was removed and transferred to fresh micro-centrifuge tubes for storage at -80°C.

The remaining pellet was washed three times in buffer A with brief centrifugation (20,000 g for 10 seconds) to remove any residual cytosolic supernatant and then resuspended in buffer B (20mM HEPES (pH 7.9), 450mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT) supplemented with protease inhibitors on the day (0.1mM PMSF, 10μg/μl soybean trypsin inhibitor and 10μg/μl benzamidine). Each sample was incubated on ice for 15 minutes with brief vortexing every 5 minutes and then centrifuged at 13,000 g for 5 minutes at 4°C. The supernatant containing the nuclear fraction was transferred to fresh micro-centrifuge tubes and stored at -80°C. Protein concentrations were measured using Bradford's assay and samples analysed by SDS-PAGE and immunoblotting.

2.2.13 Statistical Analysis

All statistical analyses were performed using Student's two-tailed unpaired *t*-test as described in Excel 2007 software.

Chapter 3

Establishing the cell systems, including the tools and conditions to use for the further examination of the working hypothesis

3. 1 Introduction

The endothelium is no longer considered an inert layer of cells lining the vasculature, but crucially an active layer of cells with many regulatory roles. A healthy endothelium presents a non-adhesive and anti-thrombotic surface, which maintains vascular tone. Impaired vasodilation is considered the hallmark of endothelial dysfunction and reflects impairment of other endothelium functions, such as the endothelium's anti-inflammatory properties (Davignon & Ganz, 2004; Landmesser *et al.*, 2004). When damaged, the endothelium assumes a highly inflammatory phenotype that leads to localised inflammation and the development of inflammatory disease states, including sepsis (Aird, 2003), diabetic retinopathy (Hsueh & Anderson, 1992) and atherosclerosis (Ludmer *et al*., 1986).

IL-6 and leptin both signal *via* the same class of receptors, namely the class I cytokine family of receptors and both have been shown to be potent pro-inflammatory cytokines in various cells, such as HUVECs, bovine aortic endothelial cells (BAEC) and mouse macrophages (Loffreda *et al.*, 1998; Romano *et al.*, 1997; Yamagishi *et al.*, 2001). They can act as acute-phase reactants, produced at high levels during inflammation, sepsis and fever (La Cava & Matarese, 2004). Importantly, both cytokines have been shown to modulate EC function, for example, by upregulating adhesion molecules such as VCAM-1 and ICAM-1, increasing MCP-1 and endothelin-1 expression, and inducing oxidative stress (Modur *et al.*, 1997; Quehenberger *et al.*, 2002; Romano *et al.*, 1997; Yamagishi *et al.*, 2001). Additionally, both have been implicated in the development of inflammatory diseases, such as RA and atherosclerosis (Bodary *et al.*, 2005; Nishimoto & Kishimoto, 2004).

The prototypical second messenger cAMP may be considered a crucial immunomodulator. Of relevance to the present study is the role that cAMP plays in the modulation of important endothelial functions. Many studies have shown that cAMP can limit vascular permeability by enhancing barrier function and reducing pro-inflammatory effects of cytokines. For example, TNFα-, PMA- or LPS-stimulated expression of E-selectin has been shown to be inhibited by cAMP elevation in HUVECs and human lung microvascular endothelial cells (HLMECs) (Blease *et al.*, 1998; Morandini *et al.*, 1996; Pober *et al.*, 1993). Additionally, inhibition of neutrophil adherence to TNFα-stimulated HLMECs in response to

cAMP elevation has been observed (Blease *et al*., 1998). Further studies have shown that cAMP elevation decreases vascular permeability in HUVECs, and increases cortical actin. Thrombin-enhanced permeability has also been observed to be reduced by cAMP treatment in HUVECs (Cullere *et al*., 2005). Another study demonstrated decreased vascular permeability and enhanced vascular endothelial (VE) cadherin-mediated adhesion in HUVECs following cAMP elevation (Fukuhara *et al*., 2005). It is important to note that the latter two studies proposed that the signalling pathway responsible for the observed effects was independent of PKA, namely the cAMP/Epac/Rap1 pathway, which is discussed in more detail in Chapter 4. Thus, a number of studies support the notion that cAMP reduces vascular permeability, and hence maintains barrier function. Although the protective effects of cAMP have been well documented, the molecular mechanisms involved remain incompletely defined.

The SOCS family of proteins represent possible mediators of the anti-inflammatory effects of cAMP. SOCS3 is one of the better studied members of the family and has been shown to be a potent inhibitor of the IL-6 and leptin signalling pathways (Bjorbaek *et al.*, 1998; Croker *et al.*, 2003; Lang *et al.*, 2003; Mori *et al.*, 2004). Importantly, previous work in this laboratory has shown that elevation of cAMP induces a time-dependent accumulation of SOCS3 protein in HUVECs (Sands *et al*., 2006). Thus, the further investigation of this SOCS protein in the context of IL-6 and leptin signalling in endothelial cells would be of interest in terms of possibly elucidating the molecular mechanisms underlying the protective effects of cAMP.

For this research, two endothelial cell types were utilised; human umbilical vein endothelial cells (HUVECs) and an angiosarcoma-derived cell line (AS-M). HUVECs have previously been used in the Palmer lab to generate publications in the same area of work as the current study (Sands & Palmer, 2005; Sands *et al.*, 2006). HUVECs have also been widely used in the literature in the context of this research area. Thus, for consistency and as an excellent point of reference from previous and ongoing research, HUVECs provide a useful and tractable endothelial cell model to study. The second cell type AS-M is a novel endothelial cell line, which has been established from a cutaneous angisarcoma on the scalp, a rare malignant neoplasm originating in the endothelium (Krump-Konvalinkova *et al*., 2003). AS-Ms display a number of endothelial characteristics, for example, the expression of proteins known to be present predominantly in endothelial cells such

as vWF and CD31 and the induction of adhesion molecules ICAM-1, VCAM-1 and E-selectin when challenged with bacterial LPS. Furthermore, they demonstrate active uptake of acetylated LDL (acLDL), which is regarded as a typical endothelial reaction (Krump-Konvalinkova *et al.*, 2003; Voyta *et al.*, 1984). These characteristics are all comparable to those of primary isolated HUVECs. Since the establishment of this cell line, the cells have undergone at least 100 population doublings. This is an obvious advantage over primary endothelial cells, since all primary cells have a limited lifespan. Extended cultivation of endothelial cultures typically results in phenotypical cell changes. During the course of the present study, HUVECs were passaged to a maximum of 4 passages. AS-Ms in comparison underwent many more passages, representing a more tractable endothelial cell type which may be used for the generation of stable knock-ins/outs. For example, one study has used AS-Ms to knock-down the G-protein-coupled sphingosine 1-phosphate receptor (S1P1) by RNA interference. Long term silencing has enabled this group to demonstrate for the first time the involvement of S1P in key functions of endothelial cells (Krump-Konvalinkova *et al*., 2005).

Using these two cell types, the objective of this results chapter was to initially establish the cell systems, which will form the basis for further examination of the working hypothesis (Section 1.6 of the Introduction). In addition, research carried out on the immortal cell line, AS-M will characterise this cell line in the context of both cytokine (IL-6 and leptin) and cAMP signalling, since there are currently very limited studies on AS-Ms in the public domain. This would be beneficial for two reasons. Firstly, AS-Ms represent a more tractable cell system when compared to HUVECs because of the potential for stable knock in/outs. Secondly, AS-Ms are less costly than HUVECs. Results from this chapter will ultimately characterise this cell line, and establish the tools and conditions to use in both cell types for further experimentation.

3.2 Results

IL-6 and leptin signal via gp130 and OB-R homodimers respectively. IL-6 first binds to a soluble form of the receptor, sIL-6α to generate a *trans-signalling* complex that is capable of efficiently activating gp130, whereas leptin can bind directly to one of six isoforms of OB-R. However, only OB-Rb has full signalling capability, due to the presence of key phosphoTyr residues on its intracellular domain (Fruhbeck, 2006; Heinrich *et al.*, 1998). IL-6 and leptin have both been shown to activate the ERK1,2 and STAT3 pathways (Fruhbeck, 2006; Heinrich *et al.*, 1998). To compare pathway activation by both cytokines in HUVECs and AS-Ms in the present study and to identify a suitable timepoint to use in subsequent experiments, HUVECs and AS-Ms were treated with IL-6/sIL-6α or leptin over a timecourse from 0 to 60 minutes, and phosphorylation of ERK1,2 and STAT3 was assessed by immunoblotting.

In HUVECs, ERK1,2 phophorylation levels (Thr202 and Tyr204 of Erk1, Thr185 and Tyr187 of Erk2) in response to IL-6/sIL-6α peaked after 15 minutes of stimulation (12±3 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control) and declined to 40±7% (n=3) of peak levels at 60 minutes. The protein kinase (PKC) activator phorbol 12-myristate 13-acetate (PMA) was included as a positive control for ERK1,2 activation in this experiment and subsequent experiments, since PMA has been shown by others to be an efficacious activator of ERK1,2 in these cells (Sexl *et al*., 1997) (Figure 3.1). STAT3 phosphorylation at Tyr705 in response to IL-6/sIL-6α treatment displayed a similar temporal pattern of phosphorylation as ERK1,2 in these cells, peaking at 15 minutes (54±17 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control) and declining to 41±11% (n=3) of peak levels at 60 minutes (Figure 3.2). In contrast to IL-6 treatment in these cells, leptin treatment could not produce a consistent pattern of ERK1,2 phosphorylation, despite using cells of different passage numbers, and cells which had been serum-starved for greater lengths of time (data not shown). Levels of phosphorylated STAT3 however, were analysed in response to leptin treatment in HUVECs and showed a time-dependent increase in STAT3 phosphorylation, peaking at 60 minutes (16±2 fold increase over basal, $p<0.01$ n=3 *versu*s vehicle-treated control) (Figure 3.3), which is in contrast to the transient phosphorylation of ERK1,2 and STAT3 in response to IL-6/sIL-6α.

In AS-Ms, ERK1,2 phosphorylation levels following 15 minutes of IL-6/sIL-6α treatment were observed to be significantly increased over vehicle-treated control (31±11 fold, p<0.05 n=3). STAT3 phosphorylation levels following IL-6/sIL-6α stimulation peaked at 30 minutes (49±11 fold increase over basal, p<0.05 n=3 *versu*s vehicle-treated control) and declined to 70±2% (n=3) of peak levels at 60 minutes (Figure 3.5). ERK1,2 phosphorylation in response to leptin in these cells was maximal at 60 minutes (6±0.3 fold increase over vehicle, p<0.001 n=3 *versu*s vehicle-treated control) (Figure 3.6), whereas STAT3 phosphorylation levels in response to leptin peaked at 30 minutes (24±5 fold increase over vehicle, p<0.01 n=3 *versu*s vehicle-treated control) and declined to 81±22% of peak levels by 60 minutes (Figure 3.7). Thus, these results demonstrate that IL-6/sIL-6α is capable of activating both the ERK1,2 and STAT3 pathways in HUVECs and AS-Ms, whereas leptin activates ERK1,2 in AS-Ms and STAT3 in both cell types.

SOCS proteins function as classical negative feedback inhibitors of cytokine signalling, since most SOCS proteins are themselves induced by cytokines. Following the discovery of these proteins, their induction was almost exclusively attributed to the JAK/STAT pathway (Starr *et al*., 1998). This is due to the presence of putative STAT-binding elements in the promoter region of the SOCS3 gene (He *et al*., 2003). SOCS3 is thus a well characterized STAT3 target gene and this was exploited in the present study for the initial examination of SOCS3 expression in the cell system following IL-6/sIL-6α or leptin treatment (Bjorbaek *et al.*, 1998; Dunn *et al.*, 2005; Starr *et al.*, 1997). Results from this would effectively test the antibodies and conditions to use for detection of SOCS3, and would contribute towards the validation of the cell system. Additionally, detection of SOCS3 in response to IL-6/sIL-6α or leptin treatment may also contribute towards the understanding of the observed transient signalling detailed earlier in this Results section. HUVECs or AS-Ms were treated with IL-6/sIL-6α or leptin over 0-90 minutes. In HUVECs, SOCS3 protein began to accumulate at 60 minutes and peaked at 90 minutes in response to IL-6/sIL-6α (21±8 fold increase over vehicle, n=3) (Figure 3.8) and leptin (9±4 fold increase over vehicle, n=3) (Figure 3.9). The same pattern of SOCS3 expression was observed in AS-Ms, whereby SOCS3 started to accumulate at 60 minutes and peaked at 90 minutes in response to IL-6/sIL-6α (64±35 fold over vehicle, n=3) (Figure 3.10) and leptin (34±5 fold increase over vehicle, p<0.01 n=3 *versu*s vehicle-treated control) (Figure 3.11). Statistical

analysis revealed that SOCS3 expression was not significantly increased over vehicle-treated control ($p>0.05$) (except for SOCS3 induction in response to leptin in AS-Ms). This is potentially due to the variability in the fold stimulation.

Further studies on the regulation of SOCS proteins revealed that these proteins are not only induced by cytokines, but also by toll-like receptor (TLR) agonists (e.g. LPS, CpG-DNA), GH, prolactin, statins and importantly, cAMP (Dalpke *et al.*, 2001; Gasperini *et al.*, 2002; Lang *et al.*, 2003; Yoshimura *et al.*, 2007). To test whether or not SOCS3 could be induced by cAMP elevation in the present study, and thus initially test the hypothesis of SOCS3 involvement in the anti-inflammatory effects of cAMP, SOCS3 protein expression was examined in HUVECs and AS-Ms in response to the cAMP-elevating agents, forskolin and rolipram. The diterpene forskolin (from the Indian plant *Coleus forskolhii*) potently activates all isoforms of ACs, except for AC9 (Yan *et al*., 1998). Rolipram is a type 4 phosphodiesterase inhibitor, which inhibits the degradation of cAMP in endothelial cells (Castro *et al*., 2005). SOCS3 accumulation in response to cAMP elevation in HUVECs has already been observed in the Palmer lab (Sands *et al*., 2006). To re-affirm this finding in HUVECs and to examine SOCS3 induction in AS-Ms, since this has not previously been observed, a combination of forskolin (Fsk) and rolipram (Roli) was used to elevate cAMP levels. Treatment of HUVECs with Fsk and Roli over 0-5 hours produced a time-dependent accumulation of SOCS3 protein (Figure 3.12a [n=1]). The same pattern of accumulation was observed in AS-Ms, with greatest levels of SOCS3 protein at 5 hours (8±2 fold increase over vehicle, $p<0.05$ n=3 *versu*s vehicle-treated control) (Figure 3.12b).

cAMP has been shown to have several effectors, including PKA (Walsh *et al*., 1968), Epac (de Rooij *et al*., 1998), cAMP-regulated ion channels (Fesenko *et al*., 1985) and CNrasGEF (Pham *et al*., 2000). The best characterised of these are PKA and Epac. Although PKA has long been thought of as the sole intracellular effector of cAMP, the discovery of Epac in 1998 led to the emergence of a key player in PKA-independent effects of cAMP (Gonzalez-Robayna *et al.*, 2000; Kashima *et al.*, 2001; Laroche-Joubert *et al.*, 2002; Rangarajan *et al.*, 2003; Schmidt *et al.*, 2001). Epac is of particular interest in the present study as it has been heavily implicated in the modulation of EC function (Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005). To examine a role for Epac in the induction of SOCS3, an Epac-selective cAMP analogue, 8-(4-chloro-phenylthio)-2'-*O*-methyladenosine-

3',5'-cyclic monophosphate (8CPT-2Me-cAMP [8-pCPT]) was used (Enserink *et al*., 2002). This analogue was used at concentrations ranging from 0-300 μM to identify a suitable concentration to use in subsequent experiments. 5 hours was chosen as a suitable timepoint to treat both cell types with 8-pCPT, since this was the timepoint that exhibited greatest accumulation of SOCS3 following forskolin and rolipram treatment. In HUVECs, SOCS3 levels peaked at 200μM (7±4 fold increase over vehicle, n=3 *versu*s vehicle-treated control) (Figure 3.13). However, this was not found to be statistically significant when compared to vehicle-treated control ($p>0.05$). This is potentially due to the variability in the fold stimulation. In AS-Ms, the greatest accumulation of SOCS3 was seen at 300μM (3±1 fold increase over vehicle, $p<0.01$ n=3 *versu*s vehicle-treated control) (Figure 3.14).

Although 8-pCPT treatment was observed to induce SOCS3 expression, the results were not conclusive and they did not discount the involvement of other cAMP-dependent effectors. To further examine a role for Epac in cAMP-mediated effects, the selective PKA inhibitor H89 was used. H89 inhibits PKA *via* binding to the ATP binding sites of PKA and inhibiting the phosphorylation process (Lochner & Moolman, 2006). The use of this inhibitor could potentially discount the involvement of PKA (the other major cAMP sensor) in cAMP-mediated effects. Thus, to initially determine the effectiveness of this inhibitor in both cell types with the aim of using this inhibitor in further experiments to test the working hypothesis, cells were treated with forskolin over 0-30 minutes and the PKA-mediated phosphorylation of cAMP response element binding protein (CREB) was assessed in the presence or absence of H89. CREB is a well characterised PKA substrate and is directly phosphorylated by PKA at serine residue 133 (Mayr & Montminy, 2001). The CREB co-activators CREB-binding protein (CBP) and p300 both bind to CREB to form a CREB-CBP/p300 complex, which can then go on to bind to cAMP response elements in target genes (Mayr & Montminy, 2001). In HUVECs, phosphorylation of CREB in response to forskolin stimulation peaked at 10 minutes (18±9 fold increase over vehicle, $p<0.001$ n=3 *versu*s vehicle-treated control) and was substantially inhibited by pre-treatment with H89 (87±6% inhibition, $p<0.001$ n=3 *versu*s 10 minute forskolin-treated cells) (Figure 3.15). Similarly, levels of CREB phosphorylation in AS-Ms peaked at 10 minutes (22±0.7 fold increase over vehicle, $p<0.001$ n=3 *versu*s vehicle-treated control) and were abolished by pre-treatment with H89 (84±4% inhibition, $p<0.001$ n=3 *versu*s 10

minute forskolin-treated cells) (Figure 3.16). No significant difference was found between vehicle-treated cells and cells treated with forskolin together with H89 pre-treatment in both cell types. These observations suggest that H89 was effective at inhibiting PKA activity at the concentration used (5μM). In addition, these results also show that forskolin is an efficacious activator of PKA-mediated CREB phosphorylation, a principal downstream target of cAMP, and thus a potent elevator of cAMP at the concentration used (10μM).

To summarise the major findings of this chapter, IL-6/sIL-6α was shown to activate both ERK1,2 and STAT3 pathways in HUVECs and AS-Ms, whereas leptin activated ERK1,2 in AS-Ms and STAT3 in both cell types. A trend of SOCS3 accumulation was observed in both cell types in response to IL-6/sIL-6α and leptin. Furthermore, a time-dependent accumulation of SOCS3 in both cell types was also observed in response to Fsk and Roli. In addition, the Epac-selective cAMP analogue 8-pCPT was shown to induce SOCS3 expression in both cell types. However, the 8-pCPT results in HUVECs were not conclusive and this could potentially be due to the variability in the fold stimulation. Finally, the expression of the well characterised PKA substrate CREB was demonstrated to be inhibited by pre-treatment with the selective PKA inhibitor H89.

3.3 Discussion

Two major intracellular pathways activated by gp130 and OB-Rb are the STAT and ERK1/2 pathways (Bromberg, 2001; Fruhbeck, 2006; Heinrich *et al.*, 2003; Vaisse *et al.*, 1996; Yang & Barouch, 2007). All IL-6-type cytokines are capable of activating STAT1 and STAT3 *via* their common receptor subunit gp130, which is ubiquitously expressed. However, STAT3 appears to be the preferred STAT protein activated by IL-6. Studies have shown that of the four distal tyrosine modules of gp130 involved in STAT activation, all four stimulate STAT3 activation, whereas STAT1 is only activated by the last two distal tyrosine modules(Gerhartz *et al.*, 1996; Stahl *et al.*, 1995). Furthermore, whereas STAT3 binds to pYXXQ motifs, STAT1 is recruited to a more restricted sequence (pYXPQ) in gp130 (Gerhartz *et al.*, 1996; Heinrich *et al.*, 1998). Gene deletion studies have shown that STAT3 deletion in mouse livers abolishes a major IL-6-mediated cellular response. namely acute phase gene induction (Alonzi *et al*., 2001). Furthermore, IL-6-deficient mice demonstrated impaired acute phase gene induction, which correlated with defective STAT3 activation (Alonzi *et al*., 1998). Similarly, *in vivo* studies have demonstrated that leptin signalling *via* OB-Rb, which is expressed almost universally including expression on HUVECs (Bouloumie *et al.*, 1998; Sierra-Honigmann *et al.*, 1998), occurs principally *via* STAT3 (Bates & Myers, 2004).

Using ERK1,2 and STAT3 phosphorylation as readouts for the activation of both of these signalling pathways, the present results show that IL-6/sIL-6α is capable of similarly activating the ERK1,2 and STAT3 pathways in HUVECs and AS-Ms, whereas leptin activates ERK1,2 in AS-Ms and STAT3 in both cell types. Various lines of evidence have demonstrated ERK1,2 and STAT3 activation in response to IL-6/sIL-6α and leptin in endothelial cells (Bouloumie *et al.*, 1998; Heinrich *et al.*, 1998; Romano *et al.*, 1997). However, this is the only study that demonstrates the activation of ERK1,2 and STAT3 in response to IL-6/sIL-6α and leptin in the AS-M cell line. In HUVECs, both ERK1,2 and STAT3 phosphorylation is maximal by 15 minutes in response to IL-6/sIL-6α. This is in agreement with other studies, such as Sobota and co-workers (2008), who demonstrated maximal STAT3 activation at 15 minutes in human dermal fibroblasts following IL-6 treatment (Sobota *et al*., 2008). Interestingly, another group (Wormald *et al*., 2006) describe a bi-phasic activation of STAT3 in response to IL-6 injection in mouse macrophages, an event

that could potentially be taking place in the present study, since STAT3 phosphorylation peaks at between 15 and 30 minutes following treatment with IL-6/sIL-6α or leptin in AS-Ms and IL-6/sIL-6α in HUVECs, and declines thereafter. Indeed, longer timepoints are required to confirm the existence of this bi-phasic pattern of activation in the present study. The present pattern of rapid activation and subsequent decline of STAT3 however has also been observed for ERK1,2 activation following IL-6/sIL-6α treatment in both cell types. In contrast, ERK1,2 activation in AS-Ms following leptin treatment was more sustained, increasing over time. Bouloumie and co-workers (1998) also showed a time-dependent increase in ERK1,2 activation in response to leptin, which they observed in HUVECs (Bouloumie *et al*., 1998). In the present study, a consistent pattern of ERK1,2 activation in HUVECs could not be achieved. This could potentially be due to low levels of OB-Rb on the cell surface of HUVECs. Indeed, a number of studies examining leptin-mediated effects routinely use cells which have been transfected with either OB-Rb or a chimeric receptor comprising the erythropoietin (Epo) extracellular domain and the OB-Rb intracellular domain (ELR) (Dunn *et al.*, 2005; Myers, 2004). This chimeric receptor is principally used to avoid potential artifacts which may be introduced as a result of endogenous short forms of the leptin receptor, since EpoR is expressed on very few cells or cell lines (Myers, 2004). Additionally, ELR has been shown by some studies to be expressed more robustly than OB-Rb (Dunn *et al*., 2005). The present study uses leptin at a concentration of 125ng/ml. Although this concentration is greater than the physiological concentration of leptin, which ranges between 5 and 15ng/ml in lean subjects (Sinha *et al*., 1996), and increases in response to overfeeding, insulin, glucocorticoids, endotoxins and cytokines (Yang & Barouch, 2007), the present study does not utilise transfected cells. This is in contrast to some studies which use leptin at a higher concentration than physiological concentrations in combination with transfected cells (Dunn *et al*., 2005).

Results from the above experiments support the general consensus that cytokine signalling is typically a transient event, suggesting the involvement of negative regulatory mechanisms. In a classical negative feedback manner, SOCS induction is one such mechanism. A plethora of studies have demonstrated cytokine induction of SOCS3. Some of these cytokines include IL-2 (Cohney *et al*., 1999), leukemia inhibitory factor (Bousquet *et al*., 1999), IL-11 (Auernhammer & Melmed,

1999), IL-6 (Starr *et al*., 1997) and leptin (Bjorbaek *et al*., 1998). Additionally, there is evidence to support an early negative feedback of cytokine signalling by SOCS3 induction. For example, SOCS3 protein expression and binding to JAK2 in murine corticotroph AtT20 cells has been shown to be maximal by 60 minutes following LIF treatment. This timepoint correlates with the dephosphoylation of JAK2 and ultimately the termination of signal transduction (Bousquet *et al*., 1999). Furthermore, work carried out by Wormald and co-workers (2006) showed that SOCS3 imposed bi-phasic kinetics upon IL-6 signalling (Wormald *et al*., 2006). Rapid STAT3 phosphorylation was observed in mouse macrophages by this group in response to IL-6 injection, with levels of phosphoylation declining between 30 and 60 minutes and then accumulating again thereafter. To examine a role for SOCS3 in this biphasic pattern of expression, IL-6 signalling was analysed in SOCS3-deficient macrophages. Results revealed that in the absence of SOCS3, IL-6-mediated STAT3 activation was maximal and sustained rather than biphasic. This suggested that SOCS3 plays a role in the early phase of IL-6 signalling. (Wormald *et al*., 2006). These findings could potentially explain the pattern of STAT3 phosphorylation in HUVECs in response to IL-6/sIL-6α in the present study, since examination of SOCS3 induction in response to IL-6/sIL-6α in HUVECs showed SOCS3 accumulation at 60 and 90 minutes. This 60 minute accumulation correlates with the decline in STAT3 phosphorylation. Indeed, the detection of the SOCS3 transcript has been observed as early as 20 minutes after IL-6 injection in mouse liver, lung and spleen (Starr *et al*., 1997). SOCS3 induction in response to IL-6/sIL-6α and leptin in AS-Ms starts to accumulate at 60 minutes, with greatest levels observed at 90 minutes. Again, this induction of SOCS3 may contribute towards the suppression of IL-6- and leptin-mediated STAT3 phophorylation. Alternatively, other negative regulatory mechanisms may also be playing a part in the downregulation of early phase signalling, such as the SH2-domain containing protein tyrosine phosphatases SHP2.

Although early inhibitory feedback signalling mechanisms are of interest, the main objective for the examination of SOC3 induction in response to IL-6/sIL-6α and leptin in both cell types in the present study was to ascertain whether SOCS3 could be detected in the cell system under conditions whereby SOCS3 has been readily observed in a number of studies. Additionally, the ability of AS-Ms to induce SOCS3 should be confirmed, since studies carried in human cancer cells

have shown that the SOCS3 promotor can become inactivated as a result of methylation (Niwa *et al.*, 2005; Sutherland *et al.*, 2004; Tokita *et al.*, 2007). The present results demonstrate a trend of SOCS3 induction in response to IL-6/sIL-6α and leptin treatment in HUVECs and AS-Ms, with highest levels of expression at 90 minutes. These results corroborate with other studies, and therefore contribute towards the validation of the cell system. In addition, the results show that SOCS3 protein expression can be detected under the conditions used.

Not only are SOCS proteins induced by cytokines, but further studies have shown that SOCS1 and SOCS3 can also be induced by non-cytokine stimuli, thus providing a mechanism by which other signalling pathways can mediate the negative regulation of cytokine signalling. Dalpke and co-workers (2001) have demonstrated SOCS1 and SOCS3 induction by bacterial CpG-DNA in J774 macrophages, RAW 264.7 macrophages, mouse bone marrow-derived dendritic cells, and peritoneal macrophages (Dalpke *et al*., 2001). CpG-DNA triggers TLR-dependent signalling cascades, such as the NF-κB pathway (Hacker *et al*., 2002), which are independent of the JAK/STAT pathway (Yi & Krieg, 1998). Other stimuli which trigger similar pathways as CpG-DNA are LPS and TNFα, both of which have been shown to induce SOCS3 expression in murine macrophages (Bode *et al.*, 1999; Stoiber *et al.*, 1999). Furthermore, inhibition of the ERK1/2 pathway by treatment with the MEK inhibitor UO126 has been shown to reduce the induction of SOCS3 by CpG-DNA, suggesting the involvement of the ERK1/2 pathway in SOCS3 induction (Dalpke *et al*., 2001). This is supported by the findings that PMA induces SOCS3 expression *via* ERK1/2 activation and pre-treatment of HepG2 and COS-7 cells with PMA inhibits STAT3 activation (Terstegen *et al*., 2000). The involvement of ERK1/2 in SOCS3 induction will be discussed in more detail in Chapter 4.

Of interest to the present study is the induction of SOCS3 by cAMP-elevating agents, since this potentially represents a mechanism by which cAMP mediates its anti-inflammatory effects. Gasperini and colleagues (2002) have demonstrated the induction of SOCS3 mRNA and protein expression in response to prostaglandin E2 (PGE2), PGE1, forskolin, dibutyryl cAMP (dbcAMP) and cholera toxin in human leukocytes (Gasperini *et al*., 2002). While, Park and co-workers (2000) have shown that treatment of FRTL-5 thyroid cells with forskolin induces the expression

of SOCS1 and SOCS3 (Park *et al*., 2000). Previous work in this laboratory has demonstrated that SOCS3 is induced by forskolin and rolipram treatment in HUVECs, whereas SOCS1 is not induced in HUVECs under conditions that readily induce the expression of SOCS3 (Sands *et al*., 2006). To re-affirm these results, HUVECs were treated with forskolin and rolipram over a timecourse, and SOCS3 expression was examined. Results showed a time-dependent accumulation of SOCS3 in response to these cAMP elevating stimuli, with greatest expression at 5 hours. In AS-Ms, a similar pattern of expression was observed with greatest accumulation of SOCS3 at 5 hours. Thus, the present results not only confirm earlier results with HUVECs, but also demonstrate SOCS3 induction in AS-Ms for the first time

Until relatively recently, the main intracellular mediator of cAMP effects was generally considered to be PKA. However, numerous studies have since discovered a new cAMP sensor, so-called Epac. Due to the roles that Epac has been ascribed to with regards to endothelial barrier function (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005) it would be of interest in the present study to determine whether Epac too plays a role in the potential anti-inflammatory effects of cAMP in endothelial cells. Indeed, the finding that the SOCS3 promoter does not contain any classical cyclic AMP response elements (CREs) (He *et al*., 2003) may support a role for PKA-independent effectors of cAMP, that do not involve the PKA-mediated activation of the transcription factor CREB. Furthermore, recent work carried out by Yarwood *et al*. (2008) showed that Epac can induce SOC3 expression in HUVECs *via* activation of the C/EBP family of transcription factors (Yarwood *et al.*, 2008).

Currently, there are no pharmacological antagonists of Epac. However a selective agonist in the form of 8pCPT has been developed. 8pCPT is an Epac-Selective Cyclic AMP Analog (ESCA) which activates Epac but not PKA, due to the incorporation of a 2'-*O*-methyl substitution on the ribose ring (2'-*O*-Me-cAMP). Further work on 2'-*O*-Me-cAMP led to the finding that a parachlorophenylthio (pCPT) substitution at position 8 on the adenine moiety of 2'-*O*-Me-cAMP (8-pCPT-2'-*O*-Me-cAMP [8-pCPT]) greatly increased its affinity for Epac, and also enhanced selectivity (107 fold greater affinity for Epac1 than for PKA) and increased cell permeability (Holz *et al*., 2008).

With the use of this selective anlogue, the current results demonstrate an accumulation of SOCS3 in response to 8-pCPT treatment at concentrations of 200μM and 300μM in HUVECs and AS-Ms respectively. However, results in HUVECs were not consistent when using this analogue, which produced variability in the fold stimulation. Of interest, previous work in the lab has suggested the involvement of Epac in SOCS3 induction. For example, the accumulation of SOCS3 protein following forskolin treatment in HUVECs was not significantly inhibited by the PKA inhibitor H89, suggesting a PKA-independent induction of SOCS3 (Sands *et al*., 2006). Additionally, a constitutively active GTPase-deficient Val12Rap1a was capable of triggering SOCS3 induction, potentially suggesting the involvement of Epac (Sands *et al*., 2006). In further agreement were the findings that Rap1a siRNA and over-expression of RapGAP substantially inhibited the induction of SOCS3 by Fsk and Roli in HUVECs (Yarwood *et al.*, 2008). Furthermore, Epac siRNA abolished the ability of Fsk and Roli to induce SOCS3 (Yarwood *et al.*, 2008). Many studies have demonstrated positive results when using 8pCPT in HUVECs, at concentrations within the range of that used in the current study (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005). 8-pCPT has even been referred to as a "super activator" so-called because it activates Epac1 with greater efficacy than cAMP *in vitro* (Rehmann *et al*., 2003b).

One explanation for the variation in the induction of SOCS3 in this study may be due to 8pCPT degradation by PDEs during the 5 hour incubation period (a timepoint chosen based on the maximal accumulation of SOCS3 in response to forskolin treatment in earlier experiments). Indeed, studies which have identified the positions on cAMP that allow interaction with PDEs, describe N^1, N^6 and N^7 on the adenine moiety as particularly important for PDE interaction and the 2'-OH moiety as not required at all (Holz *et al*., 2008). Therefore, in theory, all ESCAs are capable of interacting with PDEs and undergoing degradation. To overcome this potential problem, a PDE-resistant 8pCPT has been developed (Sp-8-pCPT-2'-*O*-Me-cAMPS), but was not used in the present study.

In view of the above results with 8pCPT, other pharmacological tools were considered in the present study, namely H89. H89 belongs to the H-series of protein kinase inhibitors, developed by Hidaka and coworkers from 1977 onwards (Lochner & Moolman, 2006). It acts competitively with ATP to bind the ATP

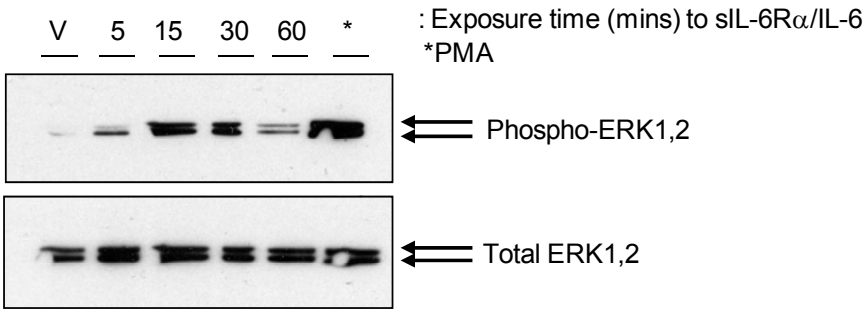
binding sites of the catalytic subunits of PKA, and inhibit the phosphorylation process. Using this inhibitor throughout the course of the study could potentially not only assess the involvement of PKA in cAMP-mediated effects, but also indirectly examine the contribution of Epac. This is based on the assumption that PKA and Epac are the 2 main cAMP effectors in the present cell system under the conditions used. The involvement of other known cAMP effectors, such as cAMP-regulated ion channels and the cyclic nucleotide rasGEF (CNrasGEF), plus the involvement of yet unknown effectors can therefore not be discounted. Furthermore, caution must be exercised when using this inhibitor as studies have shown that at a concentration of 10μM, H89 inhibits at least 8 other kinases by 80-100% (Davies *et al*., 2000). The IC_{50} values revealed that three of these kinases, namely mitogen- and stress-activated protein kinase 1 (MSK1), p70 ribosomal protein S6 kinase (S6K1) and Rho-dependent protein kinase (ROCK-II) were inhibited with a potency similar to or greater than that for PKA (Davies *et al*., 2000). In the present study, the possible inhibition of these three kinases does not appear to be of great concern, since CREB is a major substrate of PKA and results from these experiments should implicate PKA and not the other three kinases. However, MSK1 has also been shown to phosphorylate CREB. This kinase is principally a downstream target of ERK1,2 and p38 (Widmann *et al*., 1999) and although the ERK1,2 pathway and the cAMP pathway have been shown to cross-talk (which will be described in more detail in Chapter 4) cAMP-mediated CREB activation occurs predominantly *via* direct phosphorylation of serine residue 133 by the catalytic subunits of PKA (Daniel *et al*., 1998). Whilst the concentration of H89 used in the present study (5μM) may compromise its selectivity, it is nevertheless often required to achieve cell permeability, favorable stoichiometry between the inhibitor and the targets, and to compete with ATP, which is present in the cell at millimolar levels (Lochner & Moolman, 2006). Ideally, when examining the role of PKA, H89 should be used in conjunction with other PKA inhibitors, such as Rp-cAMPS and PKI analogues, or PKA-selective cAMP analogues such as 6-Bnz-cAMP. Thus, although H89 has been shown to be a potent inhibitor of PKA and has been used extensively for the assessment of the role of PKA in various biological processes, the specific inhibition of PKA is still not fully understood and this should be considered when using this inhibitor in studies.

When using this inhibitor in the present study to initially examine its effectiveness, results showed that pre-treatment of HUVECs or ASMs with H89 at a concentration of 5μM completely abolished forskolin-induced PKA-dependent CREB phosphorylation. H89 therefore appeared to be effectively inhibiting PKA at the concentration used. Indeed, many studies have used H89 at the same concentration to assess PKA involvement, including Fukuhara *et al*. (2005) to help identify the PKA-independent cAMP-Epac-Rap1 signalling pathway in endothelial barrier function (Fukuhara *et al*., 2005).

In summary, results from this Chapter demonstrate that both HUVECs and AS-Ms may be considered useful cell models for the further examination of the working hypothesis. Both cell types were capable of activating the ERK1,2 and/or STAT3 pathway(s) in response to IL-6/sIL-6α and leptin treatment. A trend of SOCS3 induction was observed in both cell types in response to IL-6/sIL-6α and leptin treatment. Furthermore, accumulation of SOCS3 over time was observed in both cell types in response to cAMP elevation. Use of the Epac-selective cAMP analogue 8-pCPT in both cell types induced SOCS3 accumulation, but produced considerable variation in the fold stimulation. In comparison, the use of H89 in both cell types demonstrated consistent inhibition of the PKA substrate CREB. Collectively, these results are important in terms of establishing the cell systems and conditions to use for further experimentation. Of particular interest are the results from AS-Ms, since there are very limited studies on AS-Ms in the public domain, and these cells represent a more tractable and less costly cell model when compared to HUVECs.

Figure 3.1 The Effect of sIL-6Rα/IL-6 Treatment on ERK1, 2 Phosphorylation in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 5, 15, 30 and 60 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with PBS alone at the same volume as sIL-6Rα/IL-6 treatment, and 1μM PMA treated cells, a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in HUVECs from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells). Basal is set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
*
: Exposure time (mins) to sIL-6Rα/IL-6
*PMA
Phospho-ERK1,2
Total ERK1,2

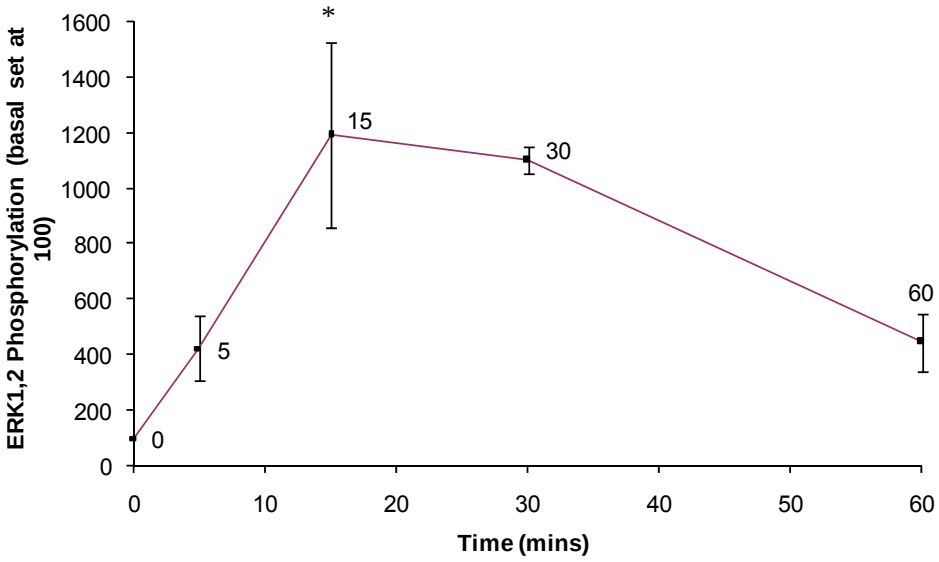
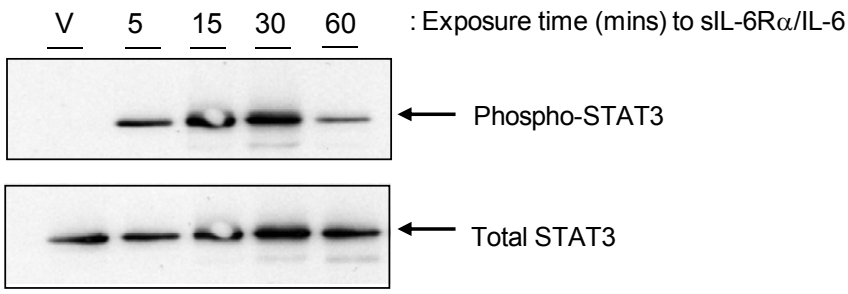

ERK1,2 Phosphorylation (basal set at 100)
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time (mins)
*
15
30
60
5
0

Figure 3.2 The Effect of sIL-6Rα/IL-6 Treatment on STAT3 Phosphorylation in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 5, 15, 30 and 60 minutes. Vehicle (V) treated cells were treated with PBS alone at the same volume as sIL-6Rα/IL-6 treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* phosphorylated STAT3 levels in vehicle-treated cells). Basal is set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
: Exposure time (mins) to sIL-6Rα/IL-6
Phospho-STAT3
Total STAT3

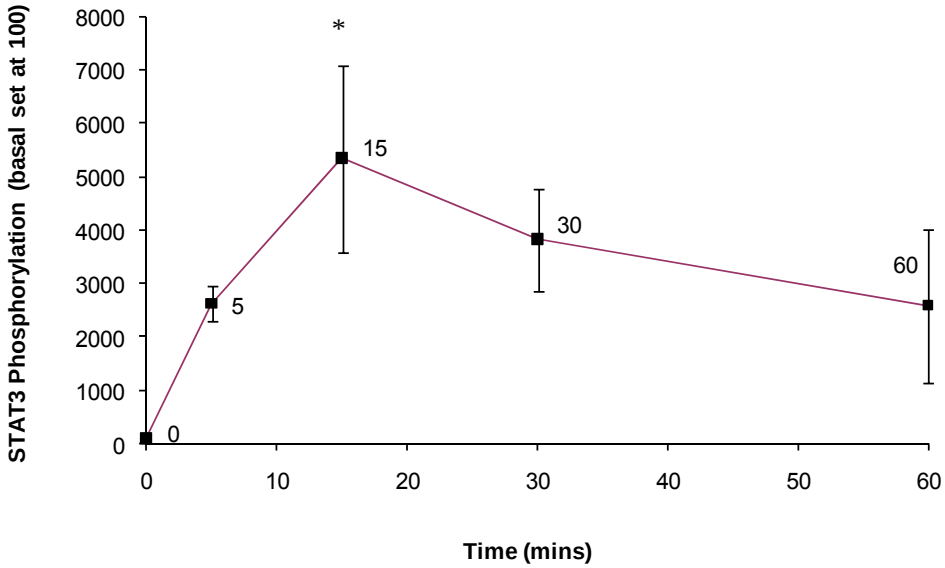
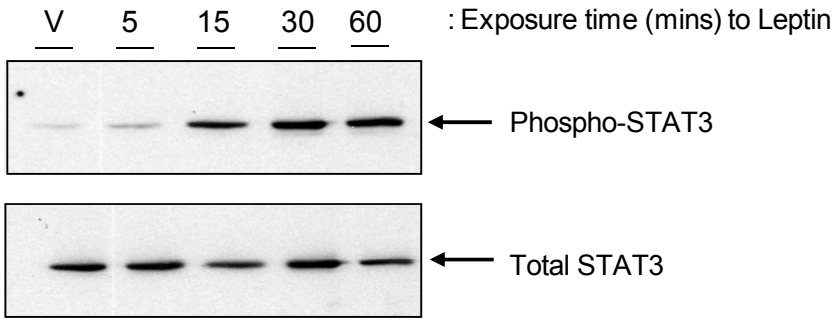

STAT3 Phosphorylation (basal set at 100)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
15
30
60
5
0
0
10
20
30
40
50
60
Time (mins)

Figure 3.3 The Effect of Leptin Treatment on STAT3 Phosphorylation in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with leptin at a concentration of 125ng/ml for 5, 15, 30 and 60 minutes. Vehicle (V) treated cells were treated with PBS alone at the same volume as leptin treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (**p<0.01 *versus* phosphorylated STAT3 levels in vehicle-treated cells). Basal is set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
: Exposure time (mins) to Leptin
Phospho-STAT3
Total STAT3

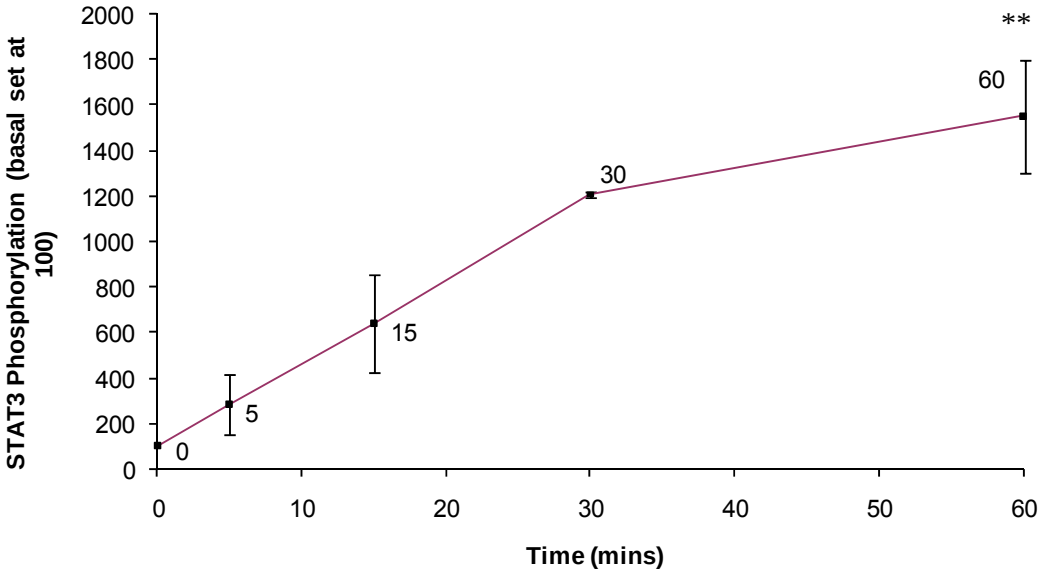
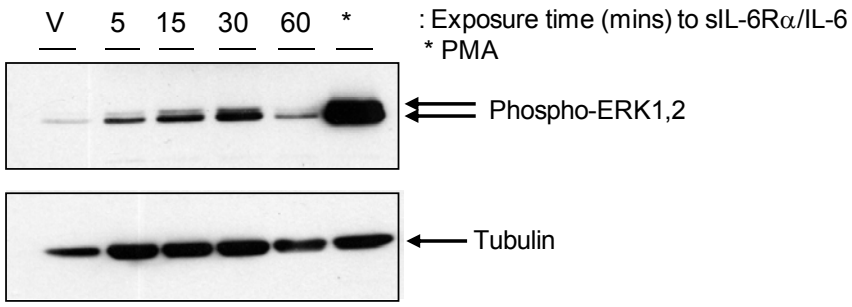

STAT3 Phosphorylation (basal set at 100)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time (mins)
0
5
15
30
60
**

Figure 3.4 The Effect of sIL-6Rα/IL-6 Treatment on ERK1, 2 Phosphorylation in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 5, 15, 30 and 60 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with PBS alone at the same volume as sIL-6Rα/IL-6 treatment, and 1μM PMA treated cells, a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells) Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
*
: Exposure time (mins) to sIL-6Rα/IL-6
* PMA
Phospho-ERK1,2
Tubulin

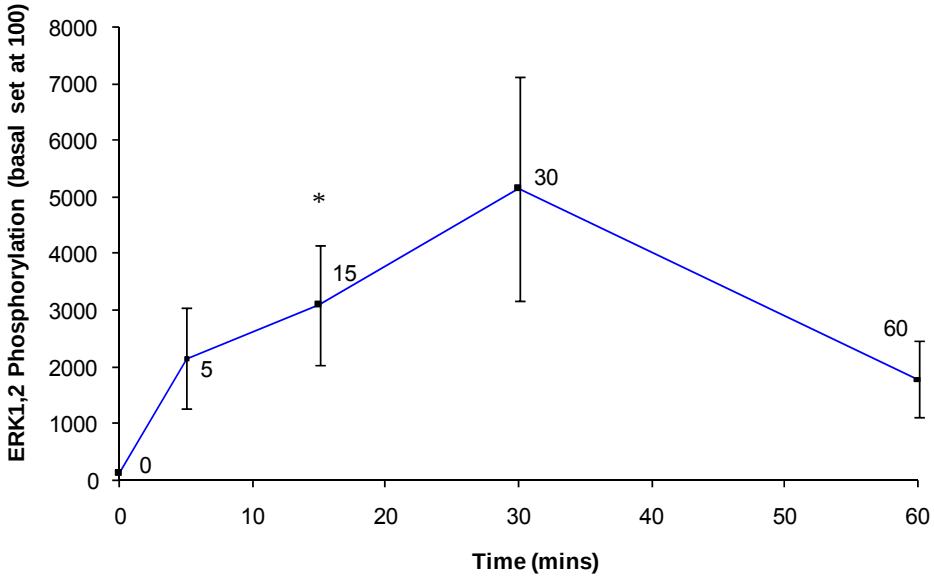
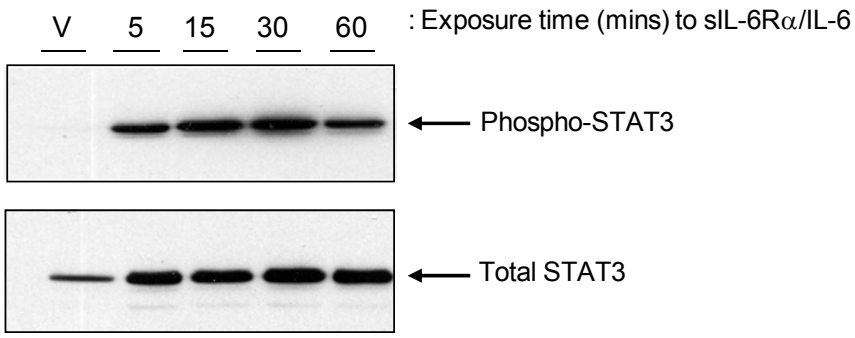

ERK1,2 Phosphorylation (basal set at 100)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
Time (mins)
0
5
*
15
30
60

Figure 3.5 The Effect of sIL-6Rα/IL-6 Treatment on STAT3 Phosphorylation in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 5, 15, 30 and 60 minutes. Vehicle (V) treated cells were treated with PBS alone at the same volume as sIL-6Rα/IL-6 treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* phosphorylated STAT3 levels in vehicle-treated cells). Basal is set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading.

.


V
5
15
30
60
: Exposure time (mins) to sIL-6Rα/IL-6
Phospho-STAT3
Total STAT3

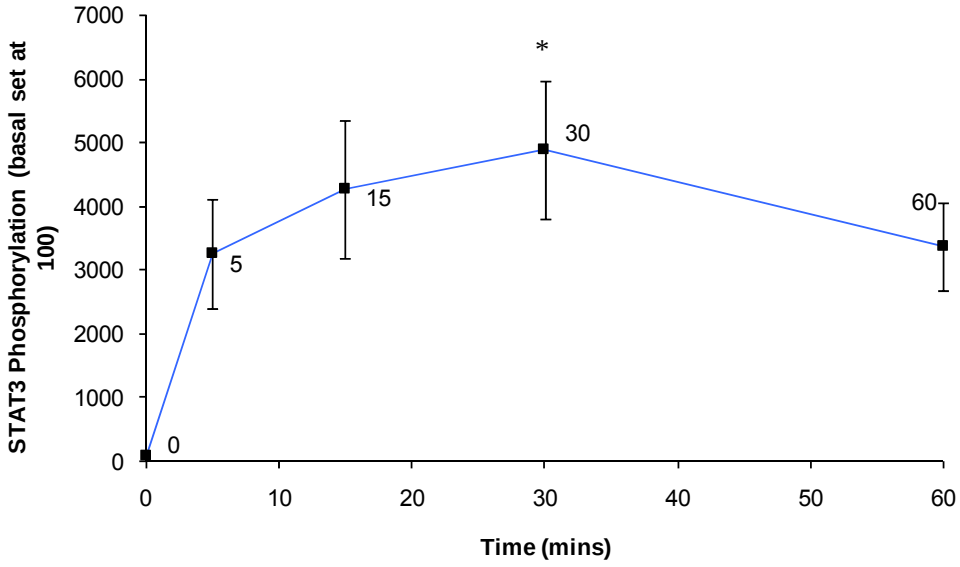
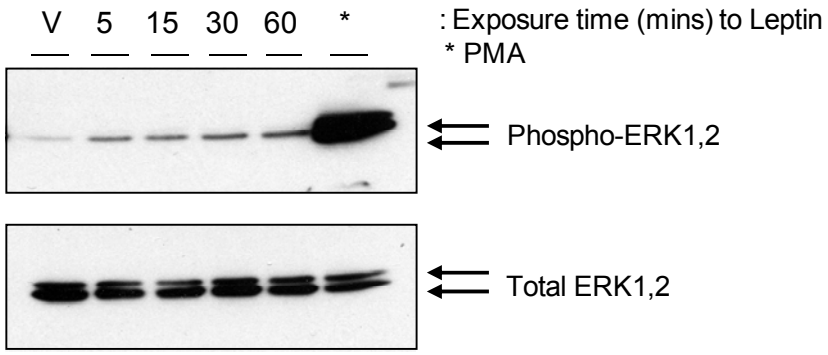

STAT3 Phosphorylation (basal set at 100)
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
Time (mins)
*
0
5
15
30
60

Figure 3.6 The Effect of Leptin Treatment on ERK1, 2 Phosphorylation in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with leptin at a concentration of 125ng/ml for 5, 15, 30 and 60 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with PBS alone at the same volume as leptin treatment, and 1μM PMA treated cells, a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells). Basal is set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading.


V 5 15 30 60 *
: Exposure time (mins) to Leptin
* PMA
Phospho-ERK1,2
Total ERK1,2

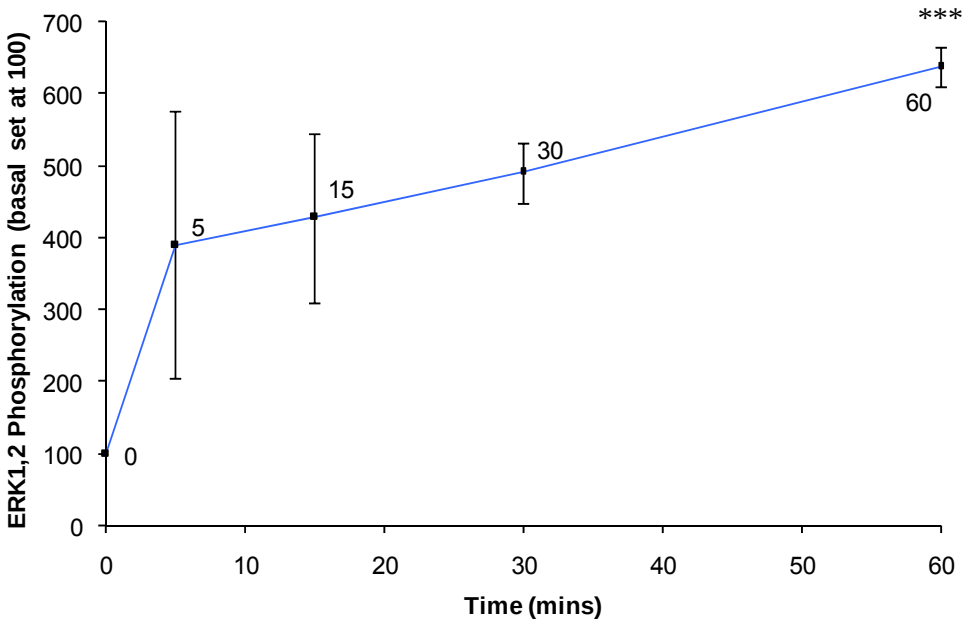
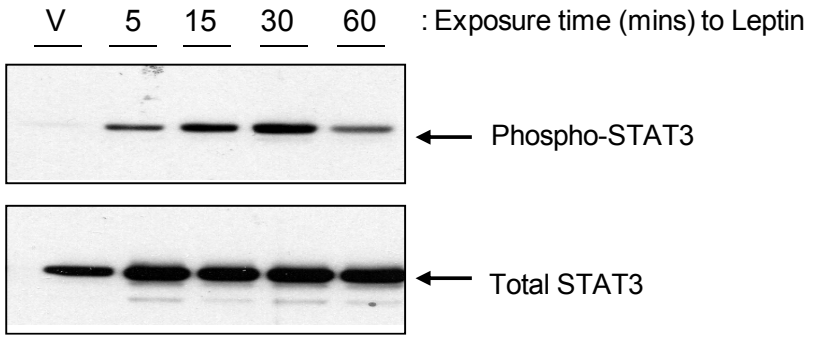

ERK1,2 Phosphorylation (basal set at 100)
Time (mins)
***
0
5
15
30
60

Figure 3.7 The Effect of Leptin Treatment on STAT3 Phosphorylation in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and serum-starved for 5 hrs. Following serum starvation, cells were treated with leptin at a concentration of 125ng/ml for 5, 15, 30 and 60 minutes. Vehicle (V) treated cells were treated with PBS alone at the same volume as leptin treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (**p<0.01 *versus* phosphorylated STAT3 levels in vehicle-treated cells. Basal is set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
: Exposure time (mins) to Leptin
Phospho-STAT3
Total STAT3

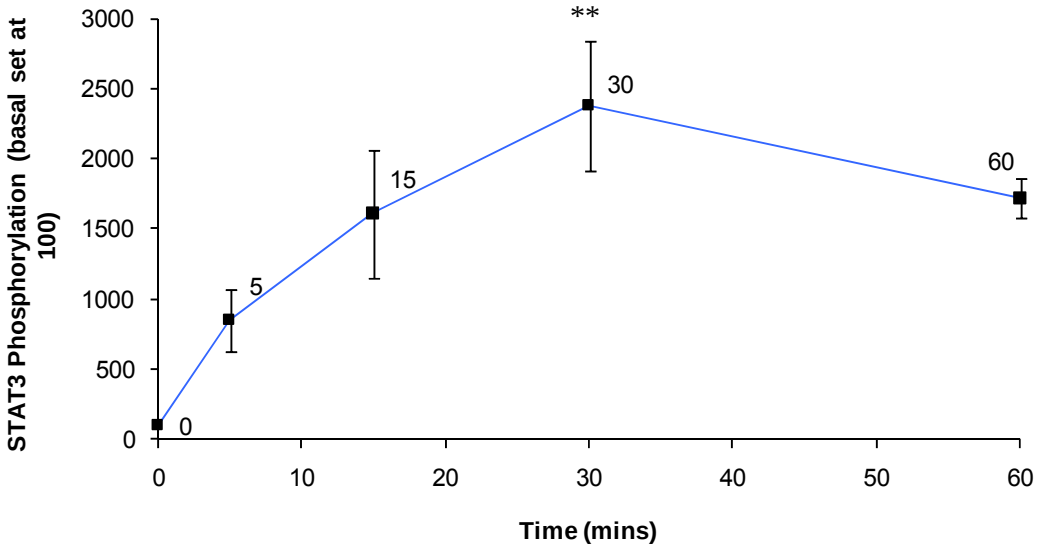
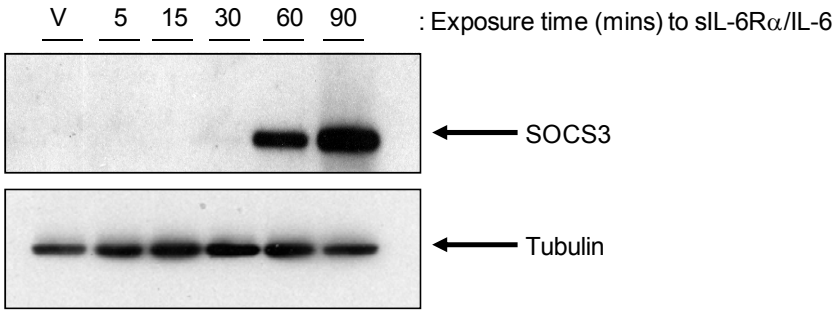

STAT3 Phosphorylation (basal set at 100)
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Time (mins)
**
0
5
15
30
60

Figure 3.8 The Effect of sIL-6Rα/IL-6 Treatment on SOCS3 Induction in HUVECs

4×10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS, before replacing the medium with serum-free medium. Cells were then treated with sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 5, 15, 30, 60 and 90 minutes, in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with PBS at the same volume as sIL-6Rα/IL-6 treatment, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in HUVECs from three experiments is presented as mean values +/- standard error (basal is set at 100). The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
90
: Exposure time (mins) to sIL-6Rα/IL-6
SOCS3
Tubulin

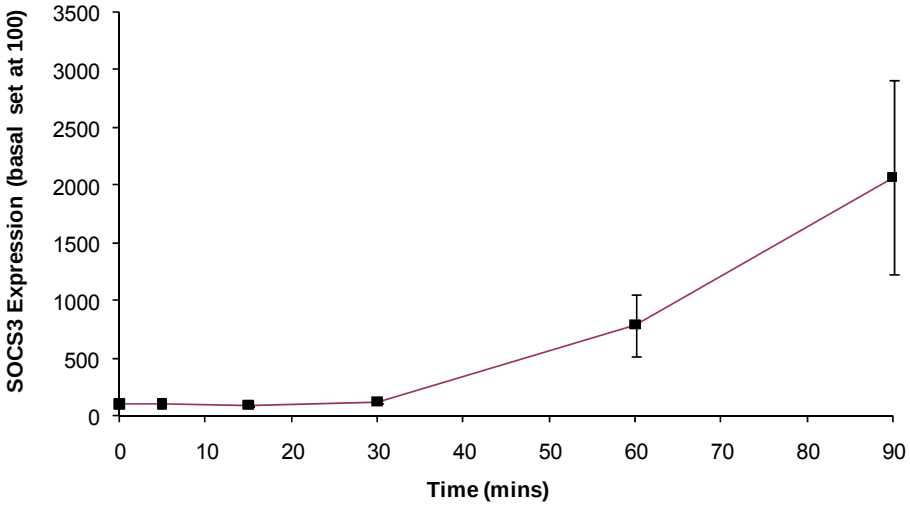
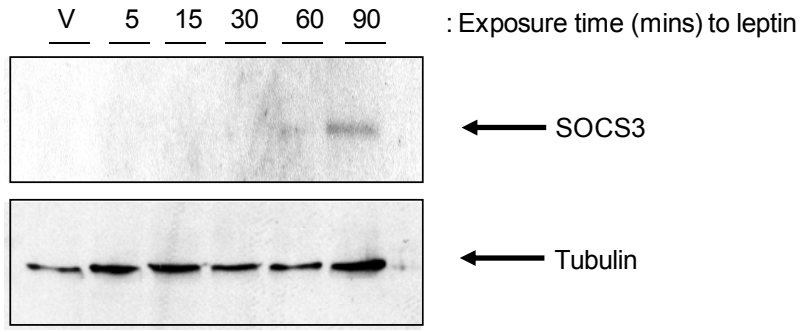

SOCS3 Expression (basal set at 100)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
Time (mins)

Figure 3.9 The Effect of Leptin Treatment on SOCS3 Induction in HUVECs

4×10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS, before replacing the medium with serum-free medium. Cells were then treated with leptin at a concentration of 125ng/ml for 5, 15, 30, 60 and 90 minutes, in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with PBS at the same volume as leptin treatment, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in HUVECs from three experiments is presented as mean values +/- standard error (basal is set at 100). The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
90
: Exposure time (mins) to leptin
SOCS3
Tubulin

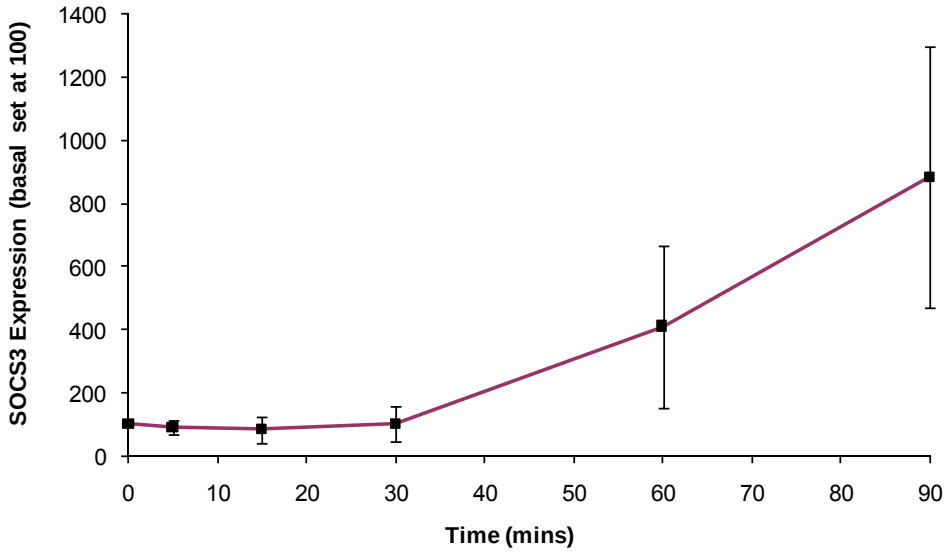
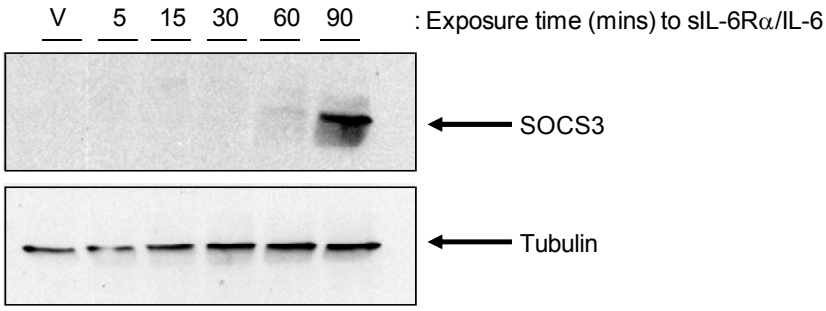

SOCS3 Expression (basal set at 100)
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
90
Time (mins)

Figure 3.10 The Effect of sIL-6Rα/IL-6 Treatment on SOCS3 Induction in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS, before replacing the medium with serum-free medium. Cells were then treated with sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 5, 15, 30, 60 and 90 minutes, in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with PBS at the same volume as sIL-6Rα/IL-6 treatment, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (basal is set at 100). The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
90
: Exposure time (mins) to sIL-6Rα/IL-6
SOCS3
Tubulin

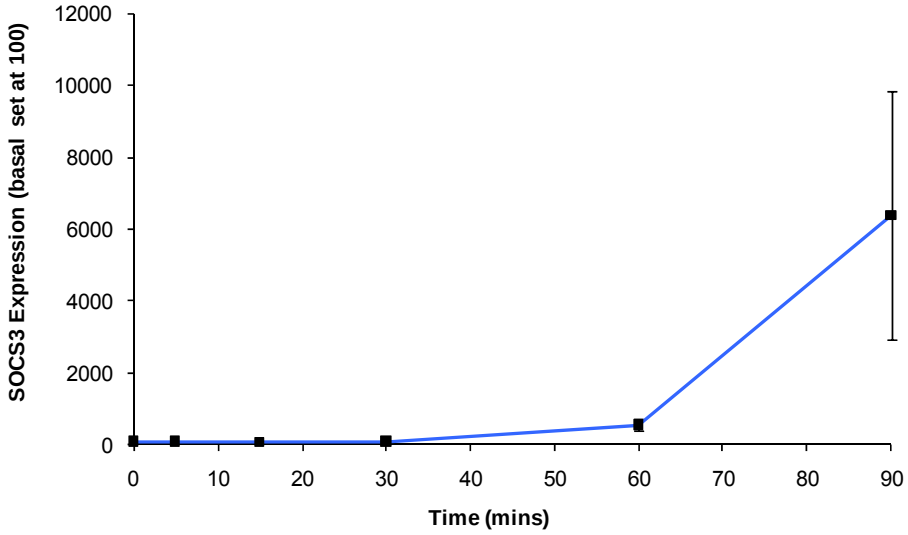
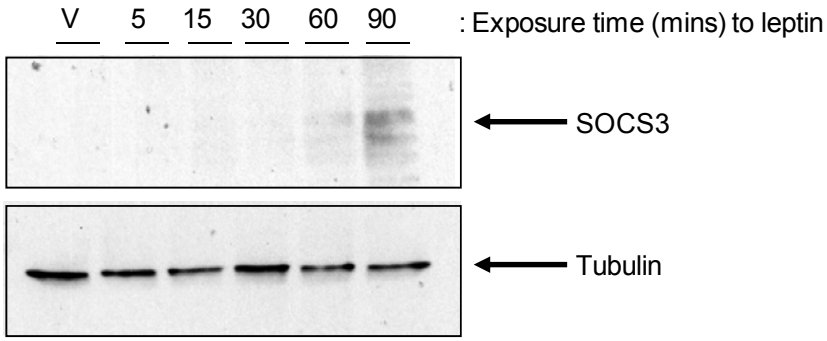

SOCS3 Expression (basal set at 100)
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40
50
60
70
80
90
Time (mins)

Figure 3.11 The Effect of Leptin Treatment on SOCS3 Induction in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS, before replacing the medium with serum-free medium. Cells were then treated with leptin at a concentration of 125ng/ml for 5, 15, 30, 60 and 90 minutes, in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with PBS at the same volume as leptin treatment, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (**p<0.01 *versus* SOCS3 expression levels in vehicle-treated cells). Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading.


V
5
15
30
60
90
: Exposure time (mins) to leptin
SOCS3
Tubulin

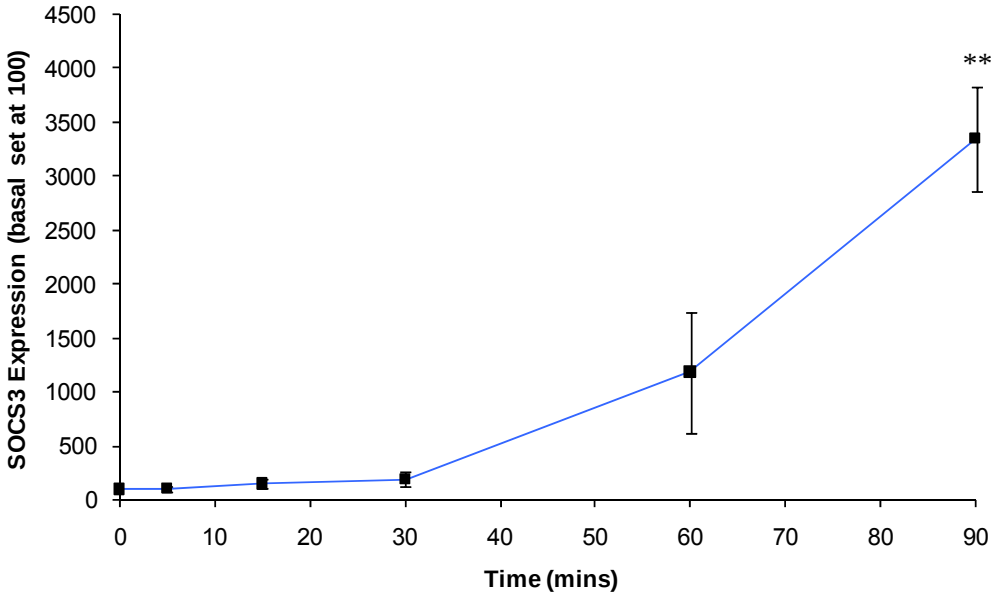
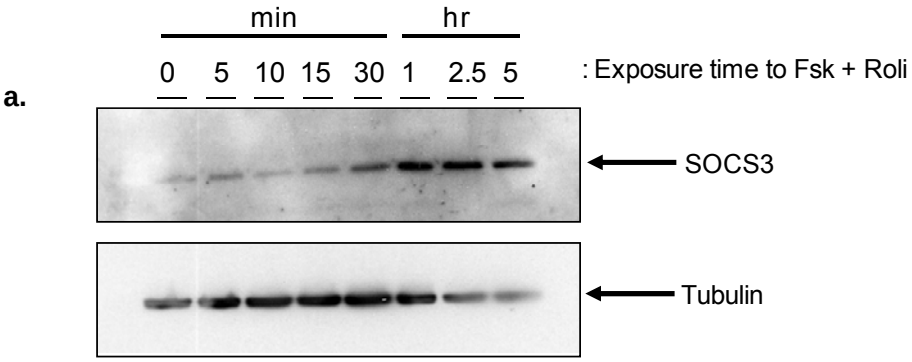
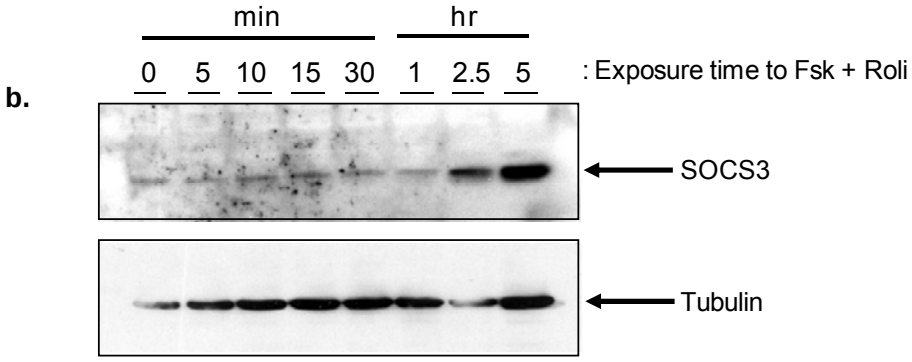

SOCS3 Expression (basal set at 100)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
Time (mins)
**

Figure 3.12 The Effect of cAMP Elevation on SOCS3 Induction in HUVECs and AS-Ms

a. 4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS before replacing the medium with serum-free medium. Cells were then treated with the adenylyl cyclase activator, forskolin (fsk) at a concentration of 10μM and the phosphodiesterase type 4 inhibitor, rolipram (roli) at a concentration of 10μM over a time course ranging from 5 minutes to 5 hours in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with ethanol and DMSO at the same volume as forskolin and rolipram treatment respectively, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. The immunoblot shows the expression of SOCS3 in HUVECs from one experiment, since previous work in the lab has demonstrated this effect consistently. The expression of the cytoskeleton protein, tubulin is also shown as an immunoblot from 1 experiment, to control for protein loading.
b. AS-Ms were treated exactly as described above. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* SOCS3 expression levels in vehicle-treated cells). Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot, to control for protein loading.


a.
min
hr
0 5 10 15 30 1 2.5 5
: Exposure time to Fsk + Roli
SOCS3
Tubulin


b.
min
hr
0 5 10 15 30 1 2.5 5
: Exposure time to Fsk + Roli
SOCS3
Tubulin

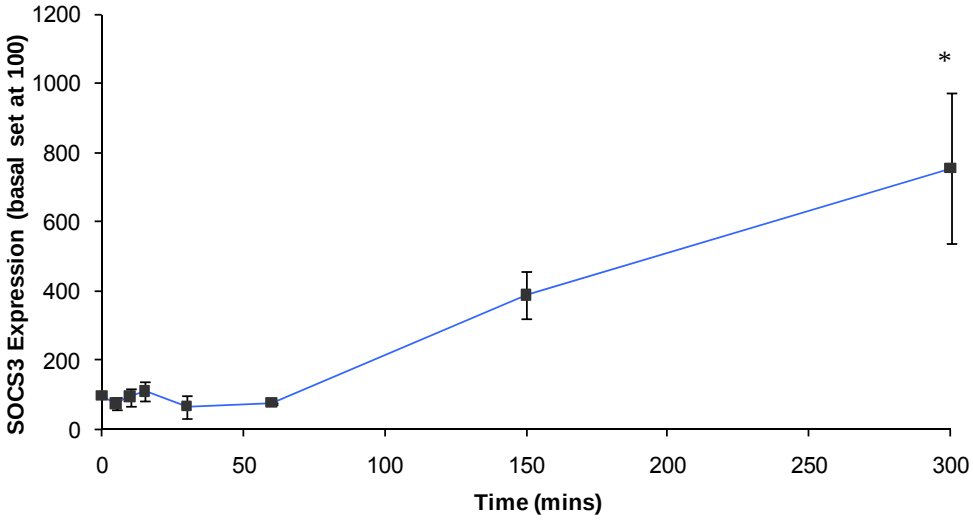
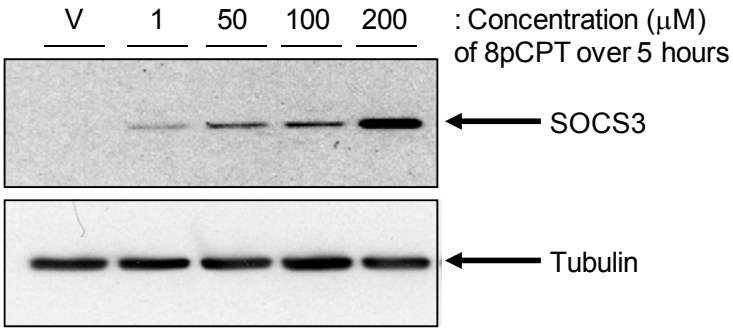

SOCS3 Expression (basal set at 100)
1200
1000
800
600
400
200
0
0
50
100
150
200
250
300
Time (mins)
*

Figure 3.13 The Effect of 8pCPT on SOCS3 Induction in HUVECs

4×10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS before replacing the medium with serum-free medium. Cells were treated with the Epac selective cAMP analogue, 8pCPT at concentrations of 1, 50, 100 and 200μM over 5 hours in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with DMSO at the same volume as 8pCPT treatment, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in HUVECs from three experiments is presented as mean values +/- standard error (basal is set at 100). The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot, to control for protein loading.


V
1
50
100
200
: Concentration (μM)
of 8pCPT over 5 hours
SOCS3
Tubulin

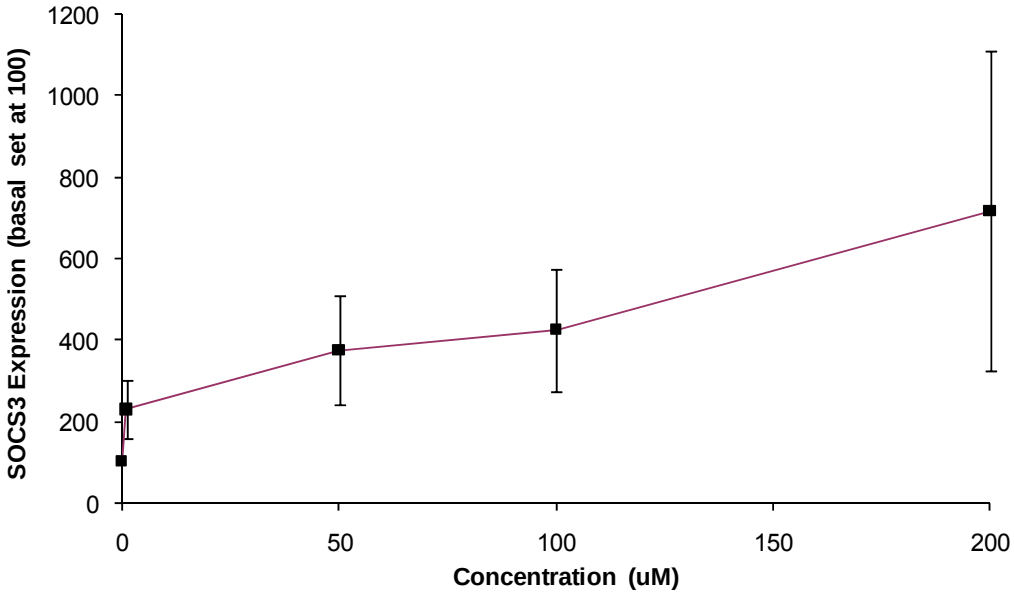
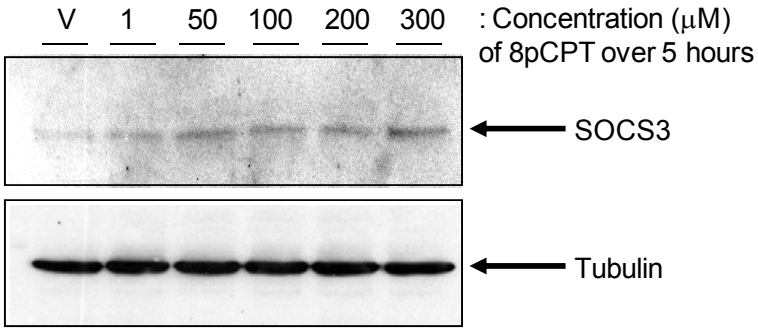

SOCS3 Expression (basal set at 100)
1200
1000
800
600
400
200
0
0
50
100
150
200
Concentration (uM)

Figure 3.14 The Effect of 8pCPT on SOCS3 Induction in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS before replacing the medium with serum-free medium. Cells were treated with the Epac selective cAMP analogue, 8pCPT at concentrations of 1, 50, 100 and 200μM over 5 hours in the presence of the proteosome inhibitor, MG132 at a concentration of 6μM. Vehicle (V) treated cells were treated with DMSO at the same volume as 8pCPT treatment, and also MG132 at a concentration of 6μM. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* SOCS3 expression levels in vehicle-treated cells). Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot, to control for protein loading.


V
1
50
100
200
300
: Concentration (μM)
of 8pCPT over 5 hours
SOCS3
Tubulin

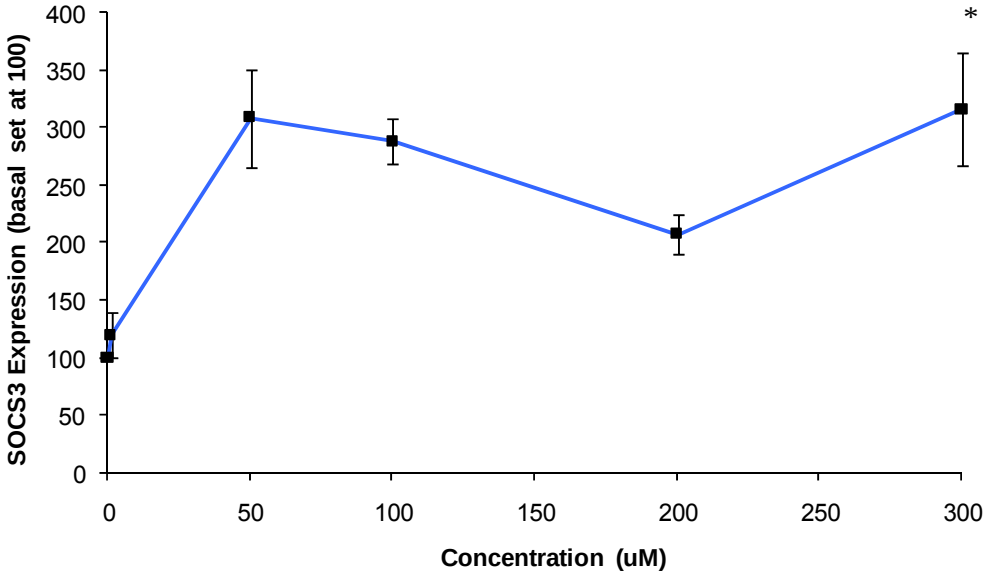
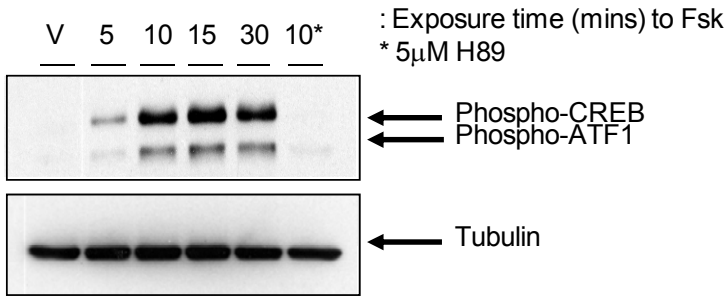

SOCS3 Expression (basal set at 100)
400
350
300
250
200
150
100
50
0
0
50
100
150
200
250
300
Concentration (uM)
*

Figure 3.15 The Effect of H89 on Forskolin Induced CREB Phosphorylation in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS before replacing the medium with serum-free medium. 1 well from each 6 well plate was pre-treated with the cAMP dependent protein kinase A inhibitor, H89 for 30 minutes at a concentration of 5μM before being treated with the adenylyl cyclase activator forskolin (fsk) at a concentration of 10μM for 10 minutes. The remaining wells were treated with fsk for 5, 10, 15 and 30 minutes. The Vehicle (V) treated cells were treated with ethanol at the same volume as fsk treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated Ser133 CREB levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated CREB levels in vehicle-treated cells, ***p<0.001 *versus* 10 minute fsk treated cells). Maximum response is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot, to control for protein loading. (ATF1 = Activating Transcription Factor 1, a member of the ATF/CREB family)


V 5 10 15 30 10*
: Exposure time (mins) to Fsk
* 5μM H89
Phospho-CREB
Phospho-ATF1
Tubulin

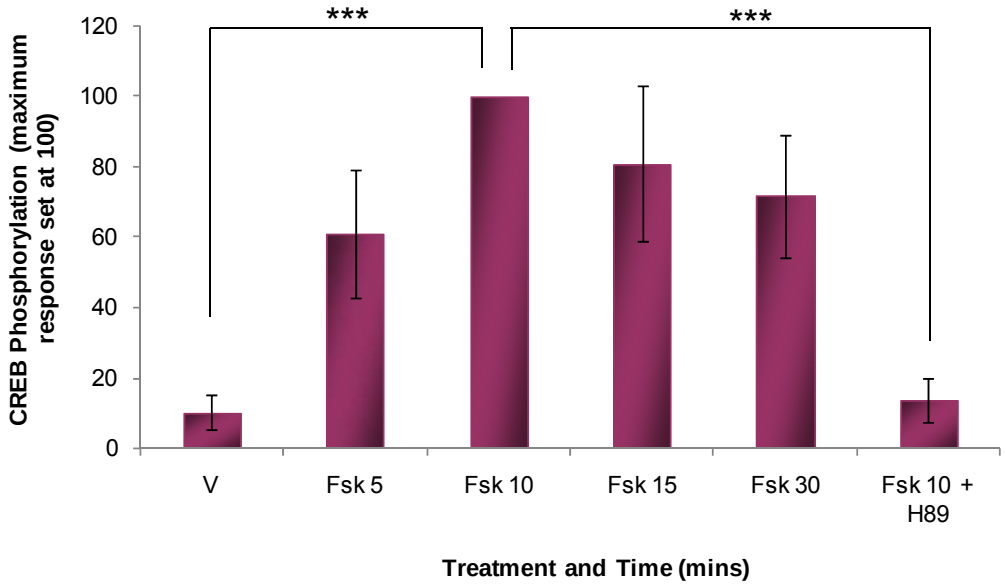
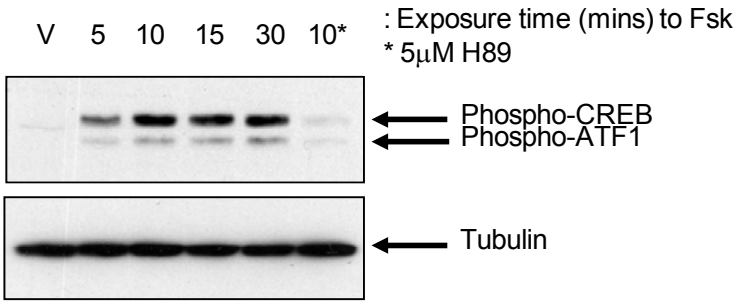
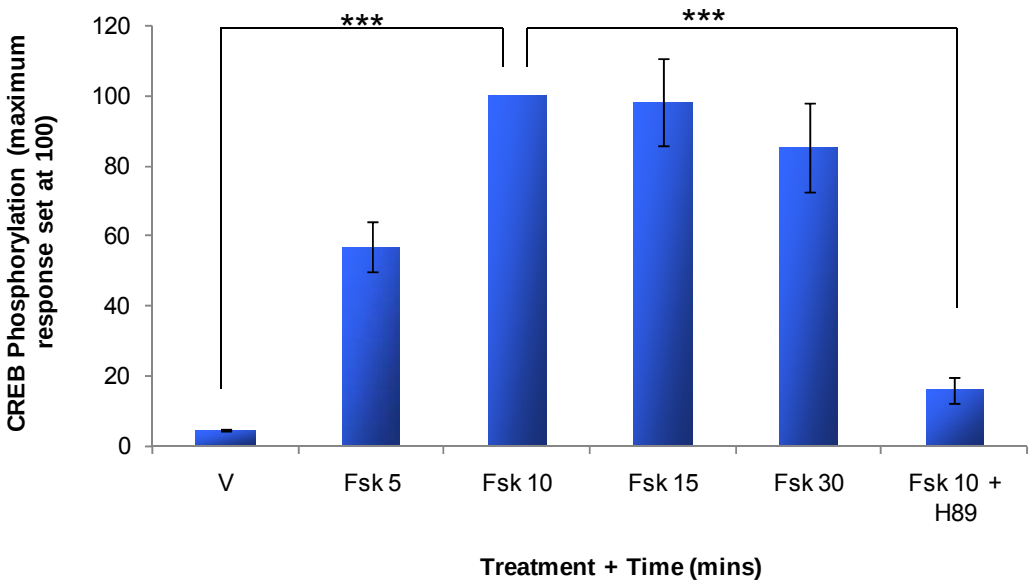

***
***
120
100
80
60
40
20
0
CREB Phosphorylation (maximum response set at 100)
V
Fsk 5
Fsk 10
Fsk 15
Fsk 30
Fsk 10 + H89
Treatment and Time (mins)

Figure 3.16 The Effect of H89 on Forskolin Induced CREB Phosphorylation in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS before replacing the medium with serum-free medium. 1 well from each 6 well plate was pre-treated with the cAMP dependent protein kinase A inhibitor, H89 for 30 minutes at a concentration of 5μM before being treated with the adenylyl cyclase activator forskolin (fsk) at a concentration of 10μM for 10 minutes. The remaining wells were treated with fsk for 5, 10, 15 and 30 minutes. The Vehicle (V) treated cells were treated with ethanol at the same volume as fsk treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated Ser133 CREB levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated CREB levels in vehicle-treated cells, ***p<0.001 *versus* 10 minute fsk treated cells). Maximum response is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot, to control for protein loading. (ATF1 = Activating Transcription Factor 1, a member of the ATF/CREB family)


V 5 10 15 30 10*
: Exposure time (mins) to Fsk
* 5μM H89
Phospho-CREB
Phospho-ATF1
Tubulin


CREB Phosphorylation (maximum response set at 100)
120
100
80
60
40
20
0
***
***
V
Fsk 5
Fsk 10
Fsk 15
Fsk 30
Fsk 10 + H89
Treatment + Time (mins)

Chapter 4

Testing the Working Hypothesis - cAMP-Mediated Inhibition of sIL-6Rα/IL-6 and Leptin Signalling in Endothelial Cells

4.1 Introduction

The downregulation of cytokine signalling has been attributed to several inhibitory mechanisms, all of which are crucial for the prevention of inappropriately sustained signalling (Wormald & Hilton, 2004). These include, but are not limited to, protein inhibitors and activators of STATs (PIAS), proteosomal degradation following Lys48 polyubiquitylation, protein tyrosine phosphatases (PTPs) and suppressor of cytokine signalling (SOCS) proteins.

The family of PIAS proteins are constitutively expressed and every member of the family has been shown to regulate STAT signalling (Shuai & Liu, 2005). In particular, PIAS3 has been shown to inhibit the DNA-binding activity of STAT3 (Chung *et al*., 1997). Interestingly, PIAS proteins have also been shown to act as E3 ligases for "small ubiquitin-related modifier" (SUMO) conjugation. For example, SUMOylation of STAT1 by PIASx-alpha has been observed. However the consequence of this modification has yet to be determined (Rogers *et al*., 2003). It was recently proposed that the phosphorylation of Ser727 on STAT1 by MAPK (p38 and ERK1/2) enhances STAT1 SUMOylation by PIAS1 (Vanhatupa *et al*., 2008), thereby implicating the MAPK pathway in the SUMOylation process. SUMO is very similar to ubiquitin, and is classed as a ubiquitin-like protein (ULP) on this basis. A STAT-interacting LIM protein (SLIM) has been shown to act as a ubiquitin E3 ligase targeting Tyr phosphorylated STAT1 and STAT4 for proteasomal-mediated degradation in T cells and STAT1 in macrophages (Gao *et al.*, 2007; Tanaka *et al.*, 2005).

PTPs comprise a large family of protein phosphatases. Of interest, PTP1B has been shown to inhibit leptin signalling via JAK2 dephosphorylation (Cheng *et al*., 2002) and TC45 has been demonstrated to suppress IL-6 induced STAT3 activation in 293T cells (Yamamoto *et al*., 2002). Of particular significance to the present study is SHP2, which not only functions as an adapter protein facilitating the activation of the ERK pathway, but also acts as a protein phosphatase, dephosphorylating Tyr-phosphorylated cytokine receptors, JAKs and STATs (Chen *et al.*, 2003; Heinrich *et al.*, 2003; Yu *et al.*, 2000). The dual function of SHP2 as a protein phosphatase and as a SH2 domain-containing protein allows this protein to potentially serve as a positive or negative regulator of IL-6 and leptin signalling. As discussed earlier in section 1.4.4 of the Introduction, it is evident that further research is required to better understand the involvement of SHP2 in these

signalling pathways. Another level of complexity to SHP2 involvement comes from the findings that SHP2 binds to the same site on gp130 and OB-Rb, namely Tyr759 and Tyr985 respectively, as SOCS3. The relative contribution of both SHP2 and SOCS3 on gp130 and OB-Rb signalling constitutes another area of research requiring more investigation.

The SOCS family of proteins are the main focus of the present study, and numerous studies have documented a role for SOCS3 in gp130 and Ob-Rb signalling, most notably studies using conditional SOCS3-deficient mice. These studies have demonstrated prolonged, unrestricted STAT3 activation in response to IL-6 and leptin in SOCS3-deficient macrophages, hepatocytes and neurons (Croker *et al.*, 2003; Mori *et al.*, 2004; Yasukawa *et al.*, 2003). There are 3 known mechanisms by which SOCS3 can downregulate cytokine signalling. The first mechanism involves SOCS3 binding to Tyr759 on gp130 and Tyr985 on OB-Rb and physically occupying the same site as other SH2-domain-containing signalling components, such as SHP2. In this way, SOCS3 competes with and inhibits other signalling pathways (De Souza *et al.*, 2002; Heinrich *et al.*, 2003; Schmitz *et al.*, 2000). The second mechanism involves the kinase inhibitory region (KIR) of SOCS3, which binds to the kinase domain of JAKs. SOCS3 acts as a pseudosubstrate, inhibiting the catalytic activity of JAKs and consequently inhibiting subsequent receptor activation (Sasaki *et al*., 1999). The third mechanism of SOCS3 inhibition involves the C terminal SOCS3 box, which can recruit an elongin B and C E3 ubiquitin-ligase complex. This complex can trigger the polyubiquitylation and proteasomal degradation of SH2 domain-bound signalling partners, such as JAK2 (Kamura *et al.*, 2004; Rui *et al.*, 2002; Zhang *et al.*, 1999a).

In view of the findings that SOCS3 is induced in response to cAMP-elevating agents, in the present study and other studies (Gasperini *et al.*, 2002; Sands *et al.*, 2006), it would be of interest to investigate the effect of cAMP elevation and SOCS3 induction on the signalling pathways of both leptin and IL-6. A cAMP-mediated, SOCS3- dependent inhibition of IL-6 and leptin signalling represents a novel mechanism by which cAMP can mediate its anti-inflammatory effects. Although the mechanism of inhibition may be novel, cross-regulation of the ERK1,2 and STAT signalling pathways by cAMP is not a new concept. Numerous studies have documented a cAMP-mediated inhibition of ERK1,2 and STAT3

signalling, some of which are detailed below. A particular focus of the present study is the cross-regulation of the ERK pathway by cAMP.

Crosstalk between the cAMP and ERK pathways has been observed since the early 1990s, whereby the cAMP analogues 8-chloro-cAMP (8-Cl-cAMP) or di-butyryl cAMP have been shown to inhibit growth factor stimulated ERK activation in fibroblasts. (Burgering *et al.*, 1993; Cook & McCormick, 1993; Wu *et al.*, 1993). In more detail, all three studies have shown that cAMP agonists are capable of inhibiting EGF-mediated MAPK in Rat-1 cells (Burgering *et al.*, 1993; Cook & McCormick, 1993; Wu *et al.*, 1993). Furthermore, Burgering and colleagues (1993) demonstrated a cAMP-mediated inhibition of PDGF- and insulin-stimulated ERK activation in NIH3T3 and Rat-1 fibroblasts (Burgering *et al*., 1993). Other cell types have also been examined in the context of cAMP-mediated inhibition, and results from these studies have shown inhibition of EGF- and insulin-stimulated ERK signalling in adipocytes and inhibition of thrombin-stimulated ERK signalling in smooth muscle cells (Osinski & Schror, 2000; Sevetson *et al.*, 1993). Thus, cAMP-mediated inhibition appears to occur in multiple cell types and affects the signalling of multiple growth factors. However, less is known about the crosstalk between cAMP and cytokine-induced ERK activation. The inhibitory effect of cAMP has been demonstrated in response to TNFα, whereby TNFα-mediated expression of E-selectin is suppressed following cAMP elevation in HUVECs and human lung microvascular endothelial cells (HLMECs) (Blease *et al.*, 1998; Morandini *et al.*, 1996). Of particular relevance to the present study is the work carried out by Sobota and co-workers (2008). These investigators demonstrated a cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 activation, but not STAT3 activation in human dermal fibroblasts and murine embryonic fibroblasts (MEFs). Additionally, the cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 activation in MEFs was shown to be independent of Epac, and dependent on PKA. (Sobota *et al*., 2008). Although not a focus of the present study, the STAT pathway has also been shown to be modulated by the cAMP pathway, wherein cAMP elevation has been shown to inhibit sIL-6Rα/IL-6-stimulated STAT3 signalling in human monocytes (Sengupta *et al*., 1996) Previous work in the Palmer lab has also demonstrated inhibition of IL-6-stimulated STAT3 phosphorylation following

chronic cAMP elevation in HUVECs and MEFs (Sands & Palmer, 2005; Sands *et al.*, 2006).

The biological relevance of this cAMP-mediated inhibition on downstream effects of ERK1,2 signalling has also been investigated. For example, numerous studies have demonstrated inhibition of proliferation in response to cAMP elevation, which is largely attributed to cAMP-mediated effects on growth factor-induced ERK1,2 activation (Bornfeldt & Krebs, 1999; Budillon *et al.*, 1999; Hecquet *et al.*, 2002; Schmitt & Stork, 2001; Tortora *et al.*, 1989). For example, cAMP elevation in smooth muscle cells has been shown to inhibit proliferation and migration by blocking growth factor receptor activation (Bornfeldt & Krebs, 1999). Proliferation and migration of smooth muscle cells have been shown to contribute towards the thickening of vessel walls, a major feature of the development of atherosclerosis. In support of these observations, the expression of many cell cycle regulatory proteins such as cyclin D and cyclin A, which are downstream targets of the ERK pathway, have been demonstrated to be inhibited by cAMP elevation (Dumaz & Marais, 2005; L'Allemain *et al.*, 1997). cAMP-mediated inhibitory effects are not limited to ERK1,2-dependent proliferation. cAMP elevation has also been shown to inhibit other ERK1,2-mediated effects, such as monocyte chemoattractant protein-1 (MCP-1) induction. MCP-1 induction by IL-6 in human dermal fibroblasts has been shown to be inhibited by prostaglandin E1 (PGE1), an initiator of cAMP signalling *via* activation of the EP2 prostaglandin G-protein-coupled receptor (GPCR). This induction of MCP-1 was shown to be ERK sensitive by demonstrating inhibition of MCP-1 following treatment with the MEK inhibitor U0126 (Sobota *et al*., 2008). In corroboration, work carried out in the Palmer lab has demonstrated inhibition of MCP-1 accumulation by sIL-6Rα/IL-6 in response to cAMP elevation by Fsk and Roli or 8pCPT treatment in HUVECs (Sands *et al*., 2006). MCP-1 plays a crucial part in the development of atherosclerosis *via* recruitment of monocytes to the endothelium at sites of vascular injury (Charo & Taubman, 2004). Of interest to the present study is the ERK transcription factor Elk-1. Elk-1 belongs to the E twenty-six (Ets) family of transcription factors, which have been shown to play roles in development, differentiation, transformation and proliferation. Elk-1, together with Net and Sap-1, forms a subfamily of the Ets family known as the ternary complex factors (TCFs) (Buchwalter *et al*., 2004). All

three TCFs characteristically form ternary complexes with serum response factor (SRF) dimers on serum response elements (SREs) present in c-fos and other early immediate gene (EIG) promoters. The TCFs represent final effectors of the MAP kinase cascade and Elk-1 is a target of all 3 MAP kinases; ERK1,2, JNK and p38 (Buchwalter *et al*., 2004). As such, Elk-1 represents a potential target of sIL-6Rα/IL-6- or leptin-stimulated ERK1,2 signalling, which could be investigated to assess the downstream effects of cAMP-mediated inhibition. The inhibition of Elk-1 by cAMP could have beneficial effects on vascular disease, such as atherosclerosis. For example, the anti-inflammatory cytokine IL-10 has been shown to suppress tissue factor (TF) expression in LPS-stimulated mouse macrophages *via* inhibition of early growth response gene 1 (Egr-1) and a MEK-ERK1,2/Elk-1 pathway (Kamimura *et al*., 2005). Elk-1, together with serum response factor has been shown to bind and activate serum response elements in the promoter of Egr-1, and Egr-1 in association with AP-1 and NFκB has been shown to activate the TF promoter. These findings together with the pro-atherogenic role of TF in thrombus formation in acute coronary syndromes, implicate Elk-1 in the disease progression of atherosclerosis (Kamimura *et al*., 2005). Of interest, Egr-1 has also been shown to be a key regulator of genes such as membrane type 1 matrix metalloproteinase and intercellular adhesion molecule 1 (ICAM-1), both of which have been demonstrated to be involved in the development of atherosclerosis (Haas *et al.*, 1999; Maltzman *et al.*, 1996).

These downstream effects are interesting in terms of contributing towards the understanding of the physiological relevance of the inhibition on sIL-6Rα/IL-6 and leptin signalling. However, the emphasis of the present study was on the further elucidation of the molecular mechanism of cAMP-mediated inhibition. A number of models exist to try to explain the inhibitory mechanism of cAMP on ERK signalling. One of which involves the direct phosphorylation of Raf by PKA. Raf serine/threonine kinases are the main effectors of Ras in the MAPK pathway. Raf-1 has been shown to be phosphorylated by PKA at 3 serine residues; S43, S233 and S259 (Dhillon *et al.*, 2002b; Dumaz *et al.*, 2002; Wu *et al.*, 1993). All three sites have been shown to block Raf-1 interaction with Ras; S43 blocks interaction *via* steric hindrance (Wu *et al*., 1993), while S233 and S259 block interaction *via* recruitment of the adapter/scaffolding proteins 14-3-3, which prevent Raf-1

recruitment to the membrane (Dumaz & Marais, 2003; Light *et al.*, 2002). Although this mode of inhibition has been well described for the cAMP-mediated inhibition of growth factor signalling, the inhibitory effects on cytokine signalling are less understood. In relation to the cAMP-mediated STAT3 inhibitory mechanism, studies have shown that expression of catalytically inactive dominant negative SHP2 mutants results in increased gp130, Jak and STAT3 phosphorylation as well as gene induction (Lehmann *et al.*, 2003; Symes *et al.*, 1997). Since SHP-2 has been shown to be positively regulated by cAMP *via* phosphorylation by PKA (Rocchi *et al*., 2000), a cAMP-mediated SHP-2-dependent inhibition of STAT3 activation describes a mode of STAT3 inhibition.

The objective of this results chapter was to initially determine whether or not cAMP elevation in HUVECs and AS-Ms exert inhibitory effects on the signalling pathways of sIL-6Rα/IL-6 and leptin. This was examined using ERK1,2 and STAT3 phosphorylation levels as end-points of sIL-6Rα/IL-6 and leptin signalling. Additionally, the downstream transcriptional activation of an ERK1,2 effector, namely Elk-1 was examined in an attempt to assess the biological consequences of cAMP-mediated inhibition of ERK1,2 activation. The focus of the present chapter however was to better understand the mechanism of inhibition, with a particular emphasis on SOCS3 as a potential mediator of the inhibitory effects (as a follow-up to previous work carried out in the Palmer lab). cAMP-mediated inhibition of ERK1,2 activation as opposed to STAT3 activation was more closely examined during this research.

4.2 Results

Inhibition of sIL-6Rα/IL-6 and Leptin Signalling by cAMP Elevation

In endothelial cells, specifically HUVECs, cAMP has been shown to play a pivotal role in reducing pro-inflammatory events and limiting vascular permeability. (Blease *et al.*, 1998; Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Morandini *et al.*, 1996). Further to the use of HUVECs in the present study, AS-Ms were utilised to potentially identify an alternative and possibly more tractable cell model when compared to HUVECs. In the present study, pre-treatment of HUVECs with the cAMP elevating agents forskolin (Fsk) and rolipram (Roli) had the effect of significantly inhibiting both ERK1,2 phosphorylation in response to sIL-6Rα/IL-6 (60±5% inhibition by Fsk + Roli *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.001$, n=3) (Figure 4.2) and STAT3 phosphorylation in response to sIL-6Rα/IL-6 (70±7% inhibition by Fsk + Roli *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.001$, n=3) (Figure 4.3). However, neither treatment alone had a significant effect on ERK1,2 or STAT3 phosphorylation in response to sIL-6Rα/IL-6. In contrast, leptin-mediated STAT3 activation could be inhibited by forskolin and rolipram in combination (62±10% inhibition by Fsk + Roli *versus* leptin-treated alone cells, $p<0.01$, n=3) as well as forskolin alone (52±16% inhibition by Fsk *versus* leptin-treated alone cells, $p<0.05$, n=3) (Figure 4.4). In AS-Ms, basal levels of ERK phosphorylation were high and neither sIL-6Rα/IL-6- or leptin-induced ERK phosphorylation were significantly above these levels ($p>0.05$). Thus, the inhibitory effect of cAMP on sIL-6Rα/IL-6 and leptin signalling could not be assessed. However, it should be noted that forskolin and rolipram in combination, as well as either treatment alone could significantly inhibit "sIL-6Rα/IL-6-treated" levels of ERK phosphorylation (76±7%, 79±8%, 80±7% inhibition by Fsk, Roli and Fsk + Roli respectively *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.001$ in all 3 comparisons, n=3) (Figure 4.5) and "leptin-treated" levels of ERK phosphorylation (80±7%, 86±2%, 87±2% inhibition by Fsk, Roli and Fsk + Roli respectively *versus* leptin-treated alone cells, $p<0.001$ in all 3 comparisons, n=3) (Figure 4.7). When examining STAT3 levels in AS-Ms, sIL-6Rα/IL-6-stimulated phosphorylation of STAT3 (29±3 fold increase over vehicle, $p<0.001$ n=3 *versus* vehicle-treated control) was modestly inhibited by forskolin and rolipram in combination (34±8% inhibition by Fsk + Roli *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.05$, n=3) and

forskolin alone (25±8% inhibition by Fsk *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.05$, n=3) (Figure 4.6). No inhibition of leptin induced STAT3 activation was observed in response to Fsk and Roli treatment (Figure 4.8).

In summary, these results show that pre-treatment of HUVECs with the cAMP elevating agents Fsk and Roli in combination for 5 hours substantially reduces the MEK-stimulated Thr and Tyr phosphorylation of ERK1,2 and the JAK stimulated Tyr phosphorylation of STAT3 in response to sIL-6Rα/IL-6. Additionally, Fsk and Roli pre-treatment in combination and Fsk treatment alone substantially inhibits leptin-induced Tyr phosphorylation of STAT3. In AS-Ms, the Tyr phosphorylation of STAT3 in response to sIL-6Rα/IL-6 was only modestly inhibited by Fsk and Roli in combination and Fsk alone. Leptin-induced Tyr phosphorylation of STAT3 was not inhibited by Fsk and Roli in combination or by either treatment alone. Levels of cAMP were not measured following 5 hours treatment with Fsk and Roli, since accumulation of SOCS3 was detected much earlier than 5 hours (Figure 3.12) and this was demonstrated to be preceeded by cAMP elevation, as determined by CREB phosphorylation (Figures 3.15 & 3.16). Lastly, Thr and Tyr phosphorylation levels of ERK1,2 in response to sIL-6Rα/IL-6 and leptin in AS-Ms were no greater than basal levels of ERK1,2 phosphorylation. Thus, the effect of Fsk and Roli on sIL-6Rα/IL-6- or leptin-stimulated ERK1,2 phosphorylation could not be assessed in AS-Ms.

Downstream Effects of cAMP-Mediated ERK1,2 Inhibition

The above results demonstrate a cAMP-mediated inhibition of sIL-6Rα/IL-6 and leptin signalling, but only demonstrate this inhibition at the level of ERK1,2 and STAT3 phosphorylation. To assess the inhibitory effect of cAMP further downstream and to potentially better understand possible biological consequences of this inhibitory effect in both cell types, a downstream effector of ERK1,2 was analysed. The well characterised ERK-responsive transcription factor Elk-1 has been demonstrated to become phosphorylated at multiple sites within the C-terminal transcriptional activation domain following binding of MAP kinases to the MAP kinase docking motif. This leads to increased Elk-1 transcriptional activation potential (Gille *et al.*, 1995; Yang *et al.*, 2002). To initially determine whether Elk-1 transcriptional activity increases following sIL-6Rα/IL-6 and leptin treatment in both cell types, a *trans*-acting reporter gene assay system was used. Cells were co-

transfected with a *trans*-activator plasmid (Gal4-Elk-1), a luciferase reporter plasmid (Gal4-luc) and a normalisation Renilla plasmid (pRL-CMV). Gal4-Elk-1 encodes a protein comprising the yeast Gal4 DNA-binding domain fused to the activation domain of Elk-1 and Gal4-luc contains the Gal4-binding element upstream of a luciferase reporter gene. Gal4-Elk-1 chimeric protein will accumulate in the cell as a result of the CMV promoter. Once activated by ERK1,2, a conformational change will allow the Gal4 DNA-binding domain to bind to the Gal4-binding element, which will drive transcription of luciferase. Both these constructs (Gal4-Elk-1 and Gal4-luc) have been used successfully to demonstrate Elk-1 activation following nociceptin treatment in CHO cells stably expressing the human opioid receptor-like 1 (ORL1) receptor (Bevan *et al.*, 1998). In the present study, cells were treated with sIL-6Rα/IL-6, leptin and PMA for 6 hrs and luminescence was read using a luminometer. The readings were expressed as average ratios of Firefly over Renilla luciferase activity from 3 independent experiments performed in triplicate. Results revealed a trend of increased Elk-1 transcriptional activity in response to PMA treatment in both HUVECs and AS-Ms. However, this increase was not found to be statistically significant over basal levels of Elk-1 transcriptional activity in both cell types. Furthermore, Elk-1 transcriptional activity following either sIL-6Rα/IL-6 and leptin treatment was either not increased or very modestly increased over basal levels of Elk1 transcriptional activity and was again found to be not statistically significant (Figure 4.9a & b). Thus, the further utilisation of these reporter gene assays to test the inhibitory effects of cAMP elevation on Elk-1 transcriptional activity was not feasible. It should, however be noted that some individual biological replicates of these assays did display statistically significant increases in Elk-1 transcriptional activity in response to PMA when compared to basal levels of Elk-1 transcriptional activity, and these increases were shown to be ERK dependent, since pre-treatment of the cells with the MEK inhibitor U0126 abolished Elk-1 activity. An example of one such replicate in HUVECs is shown in Figure 4.9c. Additionally, experiments were carried out for 12 hr treatments of sIL-6Rα/IL-6, leptin and PMA, but no increase in Elk-1 transcriptional activity over basal levels was observed (data not shown).

As a follow-up to the reporter gene assay results above, experiments were conducted to assess the translocation of ERK1,2 into the nucleus following sIL-

6Rα/IL-6 and leptin treatment. These experiments were carried out to assess whether or not the inability of sIL-6Rα/IL-6 and leptin treatment to induce Elk-1 transcriptional activity, was possibly due to poor translocation of ERK1,2 into the nucleus after stimulation with sIL-6Rα/IL-6 and leptin. HUVECs and AS-Ms were treated with sIL-6Rα/IL-6 and leptin for 30 minutes before isolating both nuclear and cytoplasmic cell fractions for detection of phosphorylated ERK1/2 and STAT3 by immunoblotting. SP1, a transcription factor, which binds to GC box promoter elements and tubulin, a cytoskeleton protein, were analysed in both fractions to confirm the isolation of nuclear and cytosolic preparations respectively. PMA-treated cells were used as a positive control in these experiments. In HUVECs, an increase in ERK1,2 phosphorylation in response to PMA treatment was observed in both the nuclear cell fraction (6±2 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control) and the cytoplasmic cell fraction (10±1 fold increase over vehicle, $p<0.001$ n=3 *versus* vehicle-treated control). Levels of ERK1,2 phosphorylation in response to sIL-6Rα/IL-6 in HUVECs were increased in the nuclear fraction only (3±0.4 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control), whereas leptin treatment did not induce ERK1,2 phosphorylation in either cytoplasmic or nuclear fractions (Figure 4.10). When examining STAT3 activation in HUVECs, levels of phosphorylated STAT3 were increased in response to sIL-6Rα/IL-6 in the cytoplasmic fraction (22±4 fold increase over vehicle, $p<0.01$ n=3 *versus* vehicle-treated control). STAT3 phosphorylation levels were also increased in response to leptin in the nuclear fraction (5±0.5 fold increase over vehicle, $p<0.01$ n=3 *versus* vehicle-treated control). In AS-Ms, ERK1,2 phosphorylation in response to PMA treatment was increased in both the cytoplasmic fraction (17±5 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control) and the nuclear fraction (16±5 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control). However, neither sIL-6Rα/IL-6- or leptin-induced ERK1,2 phosphorylation was significantly above basal levels of ERK1,2 phosphorylation in either fractions ($p>0.05$). When examining STAT3 activation in AS-Ms, an increase in STAT3 phosphorylation was observed in response to sIL-6Rα/IL-6 (8±1 fold increase over vehicle, $p<0.01$ n=3 *versus* vehicle-treated control) and leptin treatment (3±1 fold increase over vehicle, $p<0.05$ n=3 *versus* vehicle-treated control) in the nuclear fraction. Levels of STAT3 phosphorylation in

response to both sIL-6Rα/IL-6 and leptin in the cytoplasmic fractions were not significantly greater than basal levels of STAT3 phosphorylation ($p>0.05$) (Figure 4.11).

To summarise the results, which are relevant to the reporter gene assay, a marked increase in ERK1,2 phosphorylation levels in both cell fractions was observed following PMA treatment in both cell types. However, when examining sIL-6Rα/IL-6- and leptin-stimulated ERK1,2 phosphorylation in both cell types, only sIL-6Rα/IL-6-stimulated ERK1,2 phosphorylation in the nuclear fraction of HUVECs was significantly above basal levels of ERK1,2 phosphorylation.

Mechanism of Inhibition – Examining a Role for PKA and/or Epac

Although the above results were of interest in terms of the downstream effect of cAMP elevation on sIL-6Rα/IL-6 and leptin signalling, the present study aimed to focus on the molecular mechanism of cAMP inhibition. Therefore, to further investigate the working hypothesis, experiments were carried out to assess the contribution of the two major cAMP sensors; PKA and Epac in the observed inhibition of ERK1,2 and STAT3 phosphorylation following cAMP elevation. Both H89 and 8pCPT were optimised in previous experiments, which are detailed in Chapter 3. In the first instance, when examining a role for PKA in cAMP-mediated effects, pre-treatment of HUVECs with 5μM H89 followed by Fsk and Roli treatment for 5 hrs had the effect of significantly inhibiting both ERK1,2 phosphorylation (70±17% inhibition by Fsk + Roli + H89 *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.05$, n=3) and STAT3 phosphorylation (73±5% inhibition by Fsk + Roli + H89 *versus* sIL-6Rα/IL-6-treated alone cells, $p<0.001$, n=3) in response to sIL-6Rα/IL-6. The inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 phosphorylation observed in the presence of H89 was not significantly different from that seen in HUVECs pre-treated with Fsk and Roli in the absence of H89 ($p>0.05$). Indeed, the inhibition of sIL-6Rα/IL-6-stimulated STAT3 phosphorylation following Fsk and Roli treatment in the presence of H89 was even shown to be greater than the inhibition of sIL-6Rα/IL-6-stimulated STAT3 phosphorylation following Fsk and Roli treatment alone (73±5% inhibition by Fsk + Roli + H89 *versus* 50±3% inhibition by Fsk + Roli, $p<0.05$ n=3) (Figures 4.12 & 4.13). Thus, pre-treatment of HUVECs with H89 at a concentration shown to be effective in previous experiments (Figure 3.15) could not reverse the inhibitory effect of Fsk +

Roli on sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation. These results discount a role for PKA in cAMP-mediated inhibition, and may even suggest a partial repressive effect of PKA on cAMP-mediated inhibition of STAT3 activation in response to sIL-6Rα/IL-6. In comparison, the effect of H89 in AS-Ms could not be assessed, since basal levels of ERK1,2 phosphorylation were high and neither sIL-6Rα/IL-6- or leptin-induced ERK phosphorylation were significantly above these levels ($p>0.05$ n=3) (Figures 4.14 & 4.15), as observed in previous experiments (Figures 4.5 & 4.7).

When examining a role for Epac in cAMP-mediated inhibition of ERK1,2 and STAT3 phosphorylation in response to sIL-6Rα/IL-6 , HUVECs and AS-Ms were pre-treated with the Epac-selective cAMP analogue 8pCPT at 100μM for 5 hrs and ERK1,2 and STAT3 phosphorylation was assessed by immunoblotting. The experiment aimed to test whether or not 8pCPT could mimic the inhibitory effect of Fsk and Roli on sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation, and thus determine a potential role for Epac in this inhibition. The present results revealed that there was no significant difference between the levels of ERK1,2 phosphorylation in response to sIL-6Rα/IL-6 following 8pCPT treatment (with or without H89), and sIL-6Rα/IL-6-treated alone cells ($p>0.05$ n=3) (Figure 4.12). 8pCPT treatment therefore had no effect on IL-6Rα/IL-6-stimulated ERK1,2 phosphorylation and could not recapitulate the effects of Fsk and Roli treatment on sIL-6Rα/IL-6-stimulated ERK1,2 phosphorylation in HUVECs. It should be noted that the effects of 8pCPT treatment on ERK1,2 phosphorylation in HUVECs were variable, and as a consequence produced large standard error values. Variability using 8pCPT was also demonstrated in earlier experiments in Chapter 3 (Figure 3.13). Thus, a possible effect of 8pCPT on ERK1,2 phosphorylation may be masked by the variability in the fold stimulation. In comparison, when analysing STAT3 phosphorylation, 8pCPT treatment was shown to cause a 47±13% inhibition of STAT3 phosphorylation in the absence of H89 ($p<0.05$ n=3 *versus* IL-6Rα/IL-6-treated alone cells) and a 63±14% inhibition of STAT3 phosphorylation in the presence of H89 ($p<0.05$ n=3 *versus* IL-6Rα/IL-6-treated alone cells) which were comparable and not significantly different from the levels of STAT3 phosphorylation in response to sIL-6Rα/IL-6 following Fsk and Roli pre-treatment with or without H89 ($p>0.05$ n=3) (Figure 4.13). In addition, there was no

significant difference between the STAT3 phosphorylation levels following 8pCPT treatment with or without H89 pre-treatment ($p>0.05$ n=3).

Mechanism of Inhibition – Examining a Role for SOCS3

The above experiments suggest a PKA-independent inhibition of IL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation following Fsk and Roli treatment in HUVECs. Furthermore, they also suggest a potential role for Epac in the inhibition of IL-6Rα/IL-6-stimulated STAT3 activation. The present study focuses on SOCS3 as a possible mediator of these inhibitory effects and since previous experiments in the Palmer lab have suggested the involvement of Epac in SOCS3 induction (Sands *et al*., 2006), it would be of interest to further investigate a role for SOCS3 in cAMP-mediated inhibition. Results from Chapter 3 demonstrated SOCS3 induction following treatment of HUVECs and AS-Ms with Fsk and Roli (Figure 3.12). Results from the Palmer lab showed that this induction was PKA-independent, by pre-treating the cells with H89 and observing no inhibition of SOCS3 induction following Fsk treatment. Additionally, expression of a constitutively active GTPase-deficient Val12Rap1a was shown to be capable of triggering SOCS3 accumulation (Sands *et al*., 2006). Collectively, these results, together with results from Chapter 3 showing a trend of SOCS3 accumulation following 8pCPT treatment in HUVECs and AS-Ms (Figure 3.13 & 3.14) suggest an Epac-mediated SOCS3 induction, which is independent of PKA and potentially leads to the inhibition of IL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation.

To specifically test the involvement of SOCS3 in the inhibitory effects of cAMP on IL-6Rα/IL-6 and leptin signalling, siRNA was employed. In the first instance, HUVECs and AS-Ms were treated with increasing concentrations of SOCS3 siRNA and Fsk and Roli-mediated induction of SOCS3 was detected by immunoblotting. These experiments were conducted to identify a suitable concentration of SOCS3 siRNA for use in future experiments. The immunoblots show that concentrations as low as 1nM were sufficient to substantially attenuate Fsk and Roli-mediated induction of SOCS3 (Figure 4.16a. & b.). The control siRNA used in this experiment comprised of a scrambled, non-targeting sequence. Therefore, it was used as a negative control in the present experiment and all subsequent SOCS3 siRNA experiments. Results from these experiments identified 10nM as a suitable concentration to use in further SOCS3 siRNA experiments,

based on extent of knock-down of SOCS3 in both cell types, and cost effectiveness. Under conditions in which Fsk and Roli-mediated induction of SOCS3 was significantly attenuated (14±3 fold increase over vehicle in control siRNA-treated HUVECs *versus* 5±1 fold increase over vehicle in SOCS3 siRNA-treated HUVECs, $p<0.05$ n=3) (Figure 4.17), Fsk and Roli- mediated inhibition of IL-6Rα/IL-6-stimulated ERK1,2 phosphorylation was completely abolished (70±7% inhibition in control siRNA-treated HUVECs *versus* 5±18% inhibition [not significantly different from IL-6Rα/IL-6-treated alone cells] in SOCS3 siRNA-treated HUVECs, $p<0.05$ n=3) (Figure 4.18). The inhibition of IL-6Rα/IL-6-stimulated STAT3 phosphorylation following Fsk and Roli treatment in the presence of SOCS3 siRNA was also attenuated in HUVECs, although not to the same extent as the attenuation of phosphorylated ERK1,2 inhibition shown above (71±2% inhibition in control siRNA-treated HUVECs *versus* 23±3% inhibition in SOCS3 siRNA-treated HUVECs, $p<0.001$ n=3) (Figure 4.19). When examining the inhibition of leptin-stimulated STAT3 phosphorylation following Fsk and Roli treatment in HUVECs, a modest attenuation of inhibition was observed in the presence of SOCS3 siRNA (76±4% inhibition in control siRNA-treated HUVECs *versus* 56±5% inhibition in SOCS3 siRNA-treated HUVECs, $p<0.001$ n=4) (Figure 4.20). In AS-Ms, under conditions in which Fsk and Roli-mediated induction of SOCS3 was attenuated (Figure 4.21) Fsk and Roli- mediated inhibition of IL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation was not significantly attenuated by SOCS3 siRNA treatment ([ERK1,2] 70±7% inhibition in control siRNA-treated AS-Ms *versus* 54±13% inhibition in SOCS3 siRNA-treated AS-Ms, $p>0.05$ n=3 [STAT3] 37±12% inhibition in control siRNA-treated AS-Ms *versus* 36±10% inhibition in SOCS3 siRNA-treated AS-Ms, $p>0.05$ n=3) (Figures 4.22 & 4.23 respectively).

Further to these siRNA experiments, a second approach was employed to specifically test the involvement of SOCS3 in the inhibitory effects of cAMP elevation. This approach utilised W/T ($SOCS3^{+/+}$) and $SOCS3^{-/-}$ murine embryonic fibroblasts (MEFs) to compare the inhibitory effect of Fsk and Roli treatment in both cell types. To initially confirm the absence of SOCS3 in $SOCS3^{-/-}$ MEFs, cells were treated with Fsk and Roli for 5 hours prior to detection of SOCS3 expression by immunoblotting. Results revealed a 4±0.2 fold increase in SOCS3 expression

over vehicle in W/T MEFs following Fsk and Roli treatment ($p<0.001$ n=3 *versus* vehicle-treated control), but no expression of SOCS3 was detected in $SOCS3^{-/-}$ MEFs with or without Fsk and Roli treatment (Figure 4.24). When examining the inhibitory effects of cAMP in both cell types, Fsk and Roli-mediated inhibition of IL-6Rα/IL-6-stimulated ERK1,2 phosphorylation was significantly attenuated in $SOCS3^{-/-}$ MEFs (64±0.4% inhibition in W/T MEFs *versus* 19±2% inhibition in $SOCS3^{-/-}$ MEFs, $p<0.001$ n=3) (Figure 4.25). Further to this, the inhibition of IL-6Rα/IL-6-stimulated STAT3 phosphorylation was completely abolished in $SOCS3^{-/-}$ MEFs (46±10% inhibition in W/T MEFs *versus* 3±8% inhibition [not significantly different from IL-6Rα/IL-6-treated alone cells] in $SOCS3^{-/-}$ MEFs, $p<0.05$ n=3) (Figure 4.26).

Taken together, the results from the siRNA experiments and the MEFs experiments demonstrate that SOCS3 does have a role to play in the cAMP-mediated inhibition of IL-6Rα/IL-6-stimulated ERK1,2 phosphorylation in HUVECs and MEFs. Additionally, the involvement of SOCS3 in cAMP-mediated STAT3 inhibition was demonstrated in response to leptin in HUVECs and in response to IL-6Rα/IL-6 in MEFs. These results also show that the SOCS3-mediated inhibitory effects of cAMP on IL-6Rα/IL-6 signalling are not only limited to endothelial cells and may be a feature common to various cell types. On the other hand, SOCS3 siRNA appeared to have no effect on the cAMP-mediated inhibition of both ERK1,2 and STAT3 phosphorylation in response to IL-6Rα/IL-6 in AS-Ms, implying that SOCS3 is not involved in this inhibitory mechanism in AS-Ms.

Mechanism of Inhibition – Effects of PMA

To strengthen the involvement of SOCS3 in the cAMP-mediated inhibitory mechanism, experiments were conducted to investigate the level at which the inhibition was occurring within the ERK1,2 signalling pathway. PMA, a direct protein kinase C activator (PKC) is known to intercept the ERK1,2 pathway at the level of Raf *via* PKC phosphorylation of Raf-1 (Kolch *et al*., 1993). However, studies have also demonstrated a Ras-dependent activation of ERK1,2 by PMA-induced PKC activation (Verin *et al*., 2000). Additionally, other diacylglycerol/phorbol ester effectors exist, including RasGRPs, which can activate Ras independent of PKC (Brose & Rosenmund, 2002). SOCS3 intercepts the ERK1,2 pathway at the level of the receptor, upstream of Raf-1 and Ras. A very

well documented mode of cAMP inhibition on growth factor-stimulated ERK1,2 activation is the direct phosphorylation of Raf-1 by PKA at 3 serine residues; S43, S233 and S259, all of which have been shown to block Raf-1 interaction with GTP-Ras (Dhillon *et al.*, 2002b; Dumaz *et al.*, 2002; Wu *et al.*, 1993). Thus, to reinforce the notion that cAMP-mediated inhibition observed in the present study is due to SOCS3 and is independent of PKA, HUVECs were pre-treated with Fsk and Roli for 5 hours prior to 1 μM PMA treatment for 5 minutes. It was hypothesised that Fsk and Roli pre-treatment would have no effect on PMA-stimulated ERK1,2 activation if indeed the cAMP-mediated mechanism of inhibition was SOCS3-dependent and PKA-independent. However, if the well described PKA-mediated inhibition at the level of Raf-1 was involved, an inhibition of ERK1,2 phosphorylation may be observed. Further to testing these assumptions, this experiment would also test whether or not the inhibition observed in the present study was specific for the signalling pathways examined. Results revealed that there was no inhibition of PMA-stimulated ERK1,2 phosphorylation following Fsk and Roli pre-treatment in the presence or absence of H89 in HUVECs (Figure 4.27). To rule out the possibility that the concentration of PMA was masking any inhibitory effects of Fsk and Roli, varying concentrations of PMA were tested and even at concentrations as low as 0.2nM no inhibitory effects were observed (data not shown). Thus, the results supported the involvement of SOCS3 in the inhibitory mechanism and possibly further discounted a role for PKA in this mechanism. Additionally, the results also suggested a mode of inhibition which is specific for IL-6Rα/IL-6 and leptin signalling.

As a follow-up to this, experiments were conducted to examine the effect of Fsk and Roli treatment over a shorter duration prior to PMA treatment, since studies have demonstrated cAMP-mediated inhibition of phorbol ester-stimulated ERK1,2 phosphorylation following treatment with cAMP-elevating agents for 10 minutes in A14 cells (Burgering *et al*., 1993) and 15 minutes in MC3T3-E1 osteoblasts (Siddhanti *et al*., 1995). However, results revealed that there was no inhibition of PMA-stimulated ERK1,2 phosphorylation following Fsk and Roli pre-treatment for 15 minutes in the presence or absence of H89 in HUVECs (Figure 4.28). Of interest, phosphorylated ERK1,2 levels were increased above basal in response to Fsk and Roli treatment alone for 15 minutes (6±1 fold increase over vehicle,

p<0.05 n=3 *versus* vehicle-treated control). Moreover, levels of ERK1,2 phosphorylation were inhibited by pre-treating the cells with H89 (6±1 fold increase over vehicle *versus* 2±0.1 fold increase over vehicle, *p*<0.05 n=3). This is in contrast to the lack of ERK1,2 phosphorylation in response to Fsk and Roli treatment over 5 hours with or without H89 (Figure 4.27). These results are of interest since studies in the Palmer lab have demonstrated an ERK-dependent and JAK-independent induction of SOCS3 following Fsk and Roli treatment in HUVECs (Sands *et al*., 2006). The present results may therefore strengthen the involvement of ERK1,2 in SOCS3 induction.

Mechanism of Inhibition – Examining a Role for ERK1,2

However, to put this result in the context of cAMP-mediated inhibition, the next experiments investigated the effect of the MEK1,2 inhibitor U0126 on cAMP-mediated inhibition of STAT3 phosphorylation in HUVECs. Cells were pre-treated with and without U0126 prior to Fsk and Roli treatment for 5 hours. Levels of ERK1,2 and STAT3 phosphorylation were examined in response to IL-6Rα/IL-6 by immunoblotting. In the first instance, results from these experiments revealed that levels of ERK1,2 phosphorylation were not significantly above basal following IL-6Rα/IL-6 or PMA treatment in the presence of U0126 (p>0.05), which is in contrast to ERK1,2 phosphorylation levels following IL-6Rα/IL-6 and PMA treatment in the absence of U0126 (5±0.1 fold increase over vehicle, p<0.001 n=3, 12±4 fold increase over vehicle, p<0.05 n=3, respectively). Furthermore, a cAMP-mediated inhibition of IL-6Rα/IL-6-stimulated ERK1,2 phosphorylation was observed in HUVECs in the absence of U0126 (54±11% inhibition by Fsk + Roli *versus* sIL-6Rα/IL-6-treated alone cells, *p*<0.05, n=3) (Figure 4.29). Thus, under conditions in which ERK1,2 phosphorylation was abolished, the ability of Fsk and Roli to inhibit sIL-6Rα/IL-6-stimulated STAT3 phosphorylation was severely impaired (56±2% inhibition in control HUVECs *versus* sIL-6Rα/IL-6-treated alone cells, p<0.001 n=3, 12±20% inhibition in U0126-treated HUVECs *versus* sIL-6Rα/IL-6-treated alone cells, *p*>0.05 n=3) (Figure 4.30). These experiments suggest that ERK1,2 activation is required for cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated STAT3 activation in HUVECs. To further test this assumption in AS-Ms, cells were initially treated with Fsk and Roli for 15 minutes to determine whether or not cAMP elevation could activate ERK1,2. Results showed that ERK1,2 phosphorylation

was not increased over basal following Fsk and Roli treatment in AS-Ms, which is in contrast to the marked increase of ERK1,2 phosphorylation levels following PMA treatment (41±9 fold increase over vehicle, $p<0.01$ n=3) (Figure 4.31). The absence of ERK1,2 phosphorylation in these cells potentially suggests an absence of ERK1,2-mediated SOCS3 induction, and may help explain earlier results wherein SOCS3 siRNA had no effect on the inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation in AS-Ms (Figure 4.22 & 4.23). However, earlier results also demonstrated the induction of SOCS3 following Fsk and Roli treatment in AS-Ms (Figure 3.12). Thus, although Fsk and Roli treatment triggers SOCS3 induction in these cells, the induction may be completely distinct from the cAMP-mediated inhibitory effects on sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation in AS-Ms. However, it must be noted that only one time point was used to detect ERK activation following Fsk and Roli treatment in AS-Ms, and conclusions should not be made based on this result alone.

Mechanism of Inhibition – Examining a Role for Epac1

Since experiments conducted in HUVECs were generating results which were of greater relevance to the working hypothesis of the current study, the next experiments focused on HUVECs alone. Thus far, overall experiments in HUVECs have suggested a cAMP-mediated, SOCS3- and ERK1,2-dependent or SOCS3-dependent inhibition of sIL-6Rα/IL-6-stimulated STAT3 and ERK1,2 phosphorylation respectively, which is independent of PKA, and may potentially involve Epac. Since results from 8-pCPT experiments were not completely clear, another approach was used to determine the role of Epac in cAMP-mediated inhibition. This approach utilised Epac1 siRNA to examine the effect of Epac1 knock-down on sIL-6Rα/IL-6-stimulated STAT3 and ERK1,2 phosphorylation following Fsk and Roli treatment. In the first instance, a concentration of 20nM Epac1 siRNA (previously optimised in the Palmer lab) was shown to cause a 72±11% knock-down of Epac1 protein expression under basal conditions ($p<0.001$ n=5 *versus* control siRNA-treated control) (Figure 4.32). When examining the effect of this knock-down on cAMP-mediated inhibition, results revealed that Fsk- and Roli-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 phosphorylation was completely abolished in Epac1 siRNA-treated cells (60±5% inhibition in control siRNA-treated cells *versus* 7±15% inhibition [not significantly different from

IL-6Rα/IL-6-treated alone cells] in Epac1 siRNA-treated cells, $p<0.05$ n=5) (Figure 4.33). Furthermore, treatment of cells with Epac1 siRNA also had the effect of substantially attenuating the Fsk and Roli-mediated inhibition of sIL-6Rα/IL-6-stimulated STAT3 phosphorylation (41±3% inhibition in control siRNA-treated cells *versus* 13±4% inhibition in Epac1 siRNA-treated cells, $p<0.01$ n=4) (Figure 4.34). It may be worth noting in these Epac1 siRNA experiments that basal levels of ERK1,2 phosphorylation in Epac1 siRNA-treated cells were high. This may be the result of the broader effects of Epac1 knock-down in these cells. Epac has been shown to play important roles in integrin-mediated cell adhesion and cell-cell junction formation in endothelial cells (Fukuhara *et al.*, 2005; Kooistra *et al.*, 2005). Thus, disruption of these effects by Epac1 knock-down may be potentially obscuring the results. Indeed, micrograph images of the cells following siRNA transfection show that cells transfected with Epac1 siRNA look unhealthy compared with control siRNA and SOCS3 siRNA (Figure 4.35). Although this is an important point to consider when making any conclusions from the phosphorylated ERK1,2 immunblots, the basal levels of STAT3 phosphorylation following treatment of cells with Epac1 siRNA were not significantly different from the control siRNA-treated cells ($p>0.05$). Thus, the broader effects of Epac1 knock-down on the STAT3 signalling pathway appears to be unaffected.

Mechanism of Inhibition – Summary

To summarise the major findings in relation to the mechanism of inhibition, results from the present study suggest a cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation, and leptin-stimulated STAT3 activation in HUVECs. Results from AS-Ms were less clear, as basal levels of ERK1,2 phosphorylation were high and assessment of cAMP-mediated inhibition of sIL-6Rα/IL-6- and leptin-stimulated ERK1,2 activation was not feasible. Moreover, only a modest or indeed no inhibition of sIL-6Rα/IL-6- and leptin-stimulated STAT3 activation respectively was observed following Fsk and Roli treatment in these cells. The cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation observed in HUVECs appeared to be independent of PKA. Furthermore, the Fsk and Roli-mediated inhibitory effect on STAT3 activation was shown to be mimicked by the activation of Epac in HUVECs, suggesting the involvement of Epac. This result was verified by the findings that Epac1 knock-

down in HUVECs significantly impaired sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation. Further to Epac1 involvement, SOCS3 was also shown to play a crucial role in the inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation in HUVECs. However, SOCS3 siRNA results suggested no involvement of SOCS3 in the inhibition of leptin-stimulated STAT3 activation, or sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation in AS-Ms. Nevertheless, SOCS3 involvement in the inhibitory mechanism in HUVECs was strengthened by the findings that SOCS3 absence in MEFs either severely attenuated or abolished cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation, respectively. This inhibitory effect was demonstrated to be specific for the sIL-6Rα/IL-6 and leptin signalling pathway, as opposed to the PMA signalling pathway. In addition, the inhibitory effect on sIL-6Rα/IL-6-stimulated STAT3 activation (and presumably ERK1,2 activation) was shown to be dependent on ERK1,2 activation.

4.3 Discussion

4.3.1 Inhibition of sIL-6Rα/IL-6 and Leptin Signalling by cAMP Elevation

From the results of the present chapter, a potentially new pathway has been identified which inhibits cytokine receptor activation of ERK1,2 and STAT3 in endothelial cells. An inhibition of both IL-6 and leptin signalling in response to cAMP elevation in HUVECs has been observed. Of particular relevance to the present study is the inhibition of sIL-6Rα/IL-6–induced ERK1,2 activation following cAMP elevation. These findings are in agreement with other studies; for example, Sobota and co-workers have demonstrated an inhibition of IL-6-induced ERK1,2 activation following forskolin (Fsk) treatment in human dermal fibroblasts (Sobota *et al*., 2008).

In general, inhibition of ERK1,2 by cAMP has been documented in numerous cell types, including NIH3T3 fibroblasts, Rat-1 fibroblasts, adipocytes and smooth muscle cells (Burgering *et al.*, 1993; Cook & McCormick, 1993; Osinski & Schror, 2000; Sevetson *et al.*, 1993; Wu *et al.*, 1993). In comparison, cAMP elevation has also been shown to activate the ERK1,2 pathway in certain cell types, such as rat PC12 cells, N1E-115 neuroblastoma cells and melanocytes (Keiper *et al.*, 2004; Wang *et al.*, 2006). cAMP has been shown to exert these positive or negative effects on ERK1,2 signalling *via* a number of mechanisms. For example, when examining the activation of ERK1,2 by cAMP in PC12 rat pheochromocytoma cells, Wang *et al*. (2006) described a PKA-dependent activation of Rap1 *via* the Rap-GEF Crk SH3 domain Guanine nucleotide exchange (C3G). Active GTP-bound Rap1, in turn, directly binds and activates B-Raf, leading to ERK1,2 activation (Wang *et al*., 2006). A PKA-dependent activation of ERK1,2 *via* Rap1 and B-Raf activation has also been demonstrated in HEK293 cells (Schmitt & Stork, 2000). Alternatively, Keiper and co-workers have described a more indirect pathway leading to ERK1,2 activation by cAMP, which is independent of PKA. These investigators provided evidence for a Rap2b/phospholipase C (PLC)-ε/RasGRP pathway, leading to the accumulation of GTP-bound Ras and subsequent ERK1,2 activation in HEK293 cells and N1E neuroblastoma cells. Specifically, Epac-mediated activation of Rap2B was shown to activate PLC-ε, leading to an increase in intracellular calcium, and the subsequent activation of the RasGRP family of Ras-specific GEFs. This was followed by the activation of H-

Ras and finally ERK1,2 (Keiper *et al*., 2004). Yet another pathway describes how cAMP can activate ERK1,2 *via* activation of the GEF CNRasGEF, which leads to Ras activation and subsequent ERK1,2 activation in melanoma cells (Amsen *et al*., 2006).

Conversely, cAMP can inhibit ERK1,2 activation *via* several mechanisms. One of which (detailed in Chapter 4 Introduction) involves the direct phosphorylation of Raf-1 by PKA at multiple sites in its N-terminal domain, which block Raf-1 interaction with Ras (Dhillon *et al.*, 2002b; Dumaz & Marais, 2005). This mechanism has been well described for the cAMP-mediated inhibition of growth factor signalling and more recently has been proposed to be responsible for the cAMP-mediated PKA-dependent inhibition of sIL-6Rα/IL-6–induced ERK1,2 activation (Sobota *et al*., 2008). Another model of ERK1,2 inhibition by cAMP involves the phosphorylation and activation of Src-kinase by PKA, which leads to the activation of Rap1. GTP-bound Rap1 is then believed to sequester Raf-1, preventing its activation by Ras and consequently inhibiting ERK1,2 activation (Schmitt & Stork, 2001; Schmitt & Stork, 2002). Further to this, an alternative PKA-dependent inhibitory mechanism involves the induction of mitogen-activated protein kinase phosphatase 1 (MKP-1, also known as DUSP1). MKP-1 dephosphorylates MAPKs (ERK, JNK and p38 MAPKs) on tyrosine and threonine residues. MKP-1 has been shown to be induced following cAMP elevation in a PKA-dependent manner. Thus, dephosphorylation of ERK1,2 by MKP-1 represents another inhibitory mechanism (Burgun *et al.*, 2000; Sewer & Waterman, 2003). In summary, a number of mechanisms have been ascribed to the inhibition or activation of ERK1,2 in a variety of cell types, highlighting the complexity of ERK1,2 regulation by cAMP.

When examining the STAT3 pathway, a cAMP-mediated inhibition of sIL-6Rα/IL-6– and leptin-induced STAT3 activation in HUVECs was observed. These results are in contrast to the findings by Sobota and co-workers (2008); demonstrating no inhibition of STAT3 activation in response to cAMP elevation in human dermal fibroblasts (Sobota *et al*., 2008). This may be due to different experimental conditions, since these investigators treated the cells with sIL-6Rα/IL-6 for up to 60 minutes in the presence or absence of Fsk, whereas the present study pre-treated the cells with Fsk and rolipram (Roli) for 5 hours. Indeed, it could be postulated

that short-term effects of cAMP in HUVECs lead to the inhibition of ERK1,2 activation *via* the phosphorylation of Raf1 by PKA (described above), whereas long-term effects of cAMP in HUVECs result in the inhibition of both ERK1,2 and STAT3 *via* a mechanism distinct from Raf1 phosphorylation and activation. Certainly, part of the basis of this current work was to discount the involvement of the well described PKA-Raf1 inhibitory mechanism in the cAMP-mediated inhibition observed in the present study. However, work carried out by Sengupta and co-workers (1996), showed that IL-6-induced STAT3 binding to a high affinity serum-inducible element (hSIE) oligonucleotode in human mononuclear cells was inhibited following only 60 minutes pre-incubation with 8-Br-cAMP (Sengupta *et al*., 1996). This therefore also suggests the presence of cell-type specific effects of cAMP and is supported by the present results showing that STAT3 activation in response to sIL-6Rα/IL-6 was only modestly inhibited by cAMP elevation in AS-Ms and leptin-induced STAT3 activation was not inhibited at all, despite using exactly the same experimental conditions. Furthermore, the effects of cAMP on sIL-6Rα/IL-6- or leptin-induced ERK1,2 activation in AS-Ms could not be assessed as levels of ERK1,2 activation in response to both these cytokines were no greater than basal levels of ERK1,2 activation. It may be worth noting however that cAMP elevation substantially reduced "basal levels" of ERK1,2 in these cells, which has been demonstrated in other studies. For example, Wang and co-workers (2001) demonstrated inhibition of ERK1,2 phosphorylation in response to 8-CPT-cAMP, Fsk and isoproterenol when compared to basal levels of ERK1,2 phosphorylation in rat C6 glioma cells (Wang *et al*., 2001). Thus, although both cell types were endothelial in origin and both cell types were subjected to the same experimental conditions, each cell type responded differently to cAMP elevation on IL-6 and leptin signalling. It is well known that endothelial cells from different origins display variation with respect to their biochemical and immunological properties (Aird, 2005). Therefore, the differences observed between these cell types may be attributable to the different origins of these cells. Of particular relevance, AS-Ms are derived from a cutaneous angisarcoma (Krump-Konvalinkova *et al*., 2003). As a cancerous cell line, high basal levels of ERK1,2 activation may be expected in these cells.

4.3.2 cAMP-mediated PKA-independent Inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 Activation in HUVECs

Further experiments focusing on the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation in HUVECs demonstrated that this inhibition occurred *via* a mechanism independent of PKA. These results are supported by a number of studies demonstrating PKA-independent inhibitory effects of cAMP in endothelial cells. For example, cAMP elevation in HUVECs has been shown to reduce vascular permeability and enhance vascular endothelial (VE) cadherin-mediated adhesion *via* a cAMP/Epac/Rap1 pathway, which is independent of PKA (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005). Conversely, Sobota and coworkers (2008) demonstrated a cAMP-mediated inhibition of sIL-6Rα/IL-6-induced ERK1,2 activation *via* PKA-dependent phosphorylation and inhibition of Raf-1 (Sobota *et al*., 2008). Thus, results from the present study imply that the cAMP-mediated mechanism of inhibition is distinct from the well characterised PKA-dependent inhibition of Raf-1, often associated with cAMP-mediated growth factor signalling inhibition. Furthermore, these results may also discount a role for MKP-1 in the inhibition of ERK1,2, since MKP-1 has been shown to be activated by PKA. However, MKP-1 is also induced by ERK1,2 in various cell types, in an inhibitory feedback manner (Brondello *et al.*, 1997; Grumont *et al.*, 1996). Previous studies from the Palmer lab and the present study (detailed later) have implied that activation of ERK1,2 is required to observe cAMP-mediated inhibition of IL-6 signalling. Thus, cAMP-mediated and ERK1,2-dependent activation of MPK-1 could represent a mode of inhibition of sIL-6Rα/IL-6-induced ERK1,2 activation. Additionally, the H89-insensitive inhibition of sIL-6Rα/IL-6-induced STAT3 activation may also limit the involvement of SHP2 in the inhibition, since SHP2 has been shown to be positively regulated by PKA, leading to the stimulation of phosphatase activity (Rocchi *et al*., 2000). In summary, a PKA-independent inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation has been observed in HUVECs, which could potentially discount the previously described PKA-dependent mechanisms of inhibition and may implicate the other major cAMP sensor; Epac.

4.3.3 Epac-dependent & -independent Inhibition of sIL-6Rα/IL-6-induced STAT3 and ERK1,2 Activation, respectively, in HUVECs

The use of the Epac-selective cAMP analogue 8pCPT in this study showed that 8pCPT could recapitulate the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 activation, but not ERK1,2 in HUVECs. Since HUVECs have been shown to express only Epac1 and not Epac2 (Fang & Olah, 2007), it may be postulated that an Epac1-mediated inhibitory mechanism was operational. However, this did not account for the inability of 8pCPT to recapitulate cAMP-mediated inhibition of sIL-6Rα/IL-6-induced ERK1,2 activation. Previous results from Chapter 3, together with the present Chapter's results demonstrate a substantial amount of variation when using 8pCPT and this should be taken into account when making any conclusions. Indeed, varying results have been reported in the literature when using 8pCPT, in relation to ERK1,2 activation. As discussed later in the context of the present results, ERK1,2 activation appears to be necessary to observe cAMP-mediated inhibition. Previous reports in the Palmer lab have demonstrated that 8pCPT treatment in HUVECs could not activate ERK1,2 (data not published). This result is in agreement with other studies, which demonstrate Rap1 activation but not ERK1,2 activation in several cell lines, including CHO, OVCAR3, PC12, HEK293T and AtT20 (Enserink *et al.*, 2002; Wang *et al.*, 2006). Enserink *et al*. (2002) suggested that Epac-mediated Rap1 activation and cAMP-mediated ERK1,2 activation were independent processes. As such, Rap1 is incapable of activating ERK1,2. However, other studies have demonstrated that 8pCPT can activate ERK1,2 in HEK293 cells, N1E-115 neuroblastoma cells and HUVECs (Fang & Olah, 2007; Keiper *et al.*, 2004). Furthermore, Wang *et al*. (2006) proposed that the inability of Rap1 to activate ERK1,2 was not due to a property of Rap1, but rather a property of Epac. These investigators suggested that Epac-mediated ERK activation may be dependent on the localisation of Rap GEFs within the cell, and their ability to activate different pools of Rap1 (Wang *et al*., 2006). Epac1 has been shown to have a distinct perinuclear expression pattern, whereas the Rap1 GEF C3G is cytoplasmic under basal conditions and becomes recruited to the plasma membrane upon activation (Qiao *et al.*, 2002; Radha *et al.*, 2004). Rap1 activated by C3G is capable of activating ERK and this appears to be a consequence of its localisation. When the membrane targeting motif of Ki-Ras was fused to the C terminus of Epac1, Epac1 was relocated to the plasma membrane where it could activate ERK *via* a Rap1/BRaf mechanism (Wang *et al*.,

2006). Thus, variability exists in terms of 8pCPT-Rap1 regulation of ERK1,2. The present results demonstrate that 8pCPT is capable of mimicking the Fsk and Roli-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 activation, but not capable of mimicking the Fsk and Roli-mediated inhibition of sIL-6Rα/IL-6-induced ERK1,2 activation. This may suggest the involvement of two separate inhibitory mechanisms in HUVECs; an Epac1-dependent inhibition of sIL-6Rα/IL-6-induced STAT3 activation and an Epac1-independent inhibition of sIL-6Rα/IL-6-induced ERK1,2 activation. However, later experiments will disprove this assumption. A more likely explanation is experimental variation associated with this analogue. As previously mentioned in Chapter 3, 8pCPT could be getting degraded over the 5 hour incubation period. Additionally, 8pCPT has been shown to have low membrane permeability. Indeed, recently a new Epac analogue has been developed (8-pCPT-2'-O-Me-cAMP-AM), which is more efficiently delivered into cells (Vliem *et al*., 2008).

4.3.4 cAMP-mediated SOCS3-dependent & -independent Inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 Activation, and leptin-induced STAT3 Activation, respectively, in HUVECs

The PKA-independent inhibition of IL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation following cAMP elevation, and potentially Epac1-dependent inhibition of IL-6Rα/IL-6-stimulated STAT3 activation in HUVECs, did not alter the levels of total ERK1,2 and STAT3 expression. From these observations it may be postulated that a post-receptor inhibitory mechanism was operational. In line with the working hypothesis and the involvement of SOCS3 in the inhibitory mechanism, previous results from the Palmer lab have shown that Fsk-mediated induction of SOCS3 is not inhibited by H89 in HUVECs (Sands *et al*., 2006). Additionally, expression of a constitutively active GTPase-deficient Val12Rap1a was shown to be capable of triggering SOCS3 accumulation in HUVECs (Sands *et al*., 2006). Further to this, depletion of Rap1a using specific siRNA targeting Rap1a in HUVECs substantially attenuated SOCS3 induction by Fsk and Roli. Furthermore, depletion of Epac1 using siRNA in HUVECs was shown to abolish SOCS3 induction following cAMP elevation (Sands *et al*., 2006). Lastly, over-expression of RapGAP, which is known to increase the intrinsic GTPase activity of Rap1, was shown to severely attenuate the ability of Fsk and Roli, and 8-pCPT to

induce SOCS3 in HUVECs (Yarwood *et al.*, 2008). Collectively, these results together with the results from Chapter 3, which show a trend of SOCS3 accumulation following 8pCPT treatment in HUVECs and AS-Ms (Figure 3.13 & 3.14) suggest an Epac1-mediated SOCS3 induction, which is independent of PKA. The possibility of SOCS3 involvement in the cAMP-mediated inhibition of IL-6 and leptin signalling was next investigated by using specific siRNA targeting SOCS3. These experiments demonstrated that SOCS3 is involved in the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation in HUVECs. However, a role for SOCS3 in the cAMP-mediated inhibition of leptin-induced STAT3 activation in HUVECs was not demonstrated. The SOCS3 binding sites on the leptin receptor have been identified as pTyr985 and pTyr1077. The affinity of SOCS3 binding to these sites has been shown to be much lower than for pTyr757 on gp130. For example, pTyr985 has an 80-fold lower affinity for SOCS3, when compared to pTyr757 (De Souza *et al*., 2002). As an alternative to SOCS3, protein tyrosine phosphatase 1B (PTP1B) may be a strong candidate for the observed negative regulation of leptin signalling. Supporting this assumption is a study using PTP1B-deficient mice. These mice are hypersensitive to insulin and leptin, and are resistant to diet-induced obesity (Elchebly *et al*., 1999). A large body of evidence suggests that PTP1B targets leptin signaling mainly *via* JAK2 dephosphorylation and thus targets both the STAT and ERK1,2 pathways (Lavens *et al*., 2006). Interestingly, serine phosphorylation of PTP1B in response to cAMP analogues has been shown in HeLa cells (Brautigan & Pinault, 1993). Further to PTP1B, other SOCS members could be involved in the cAMP-mediated inhibition of leptin signalling. For example, SOCS7 has been shown to inhibit leptin-induced STAT3 activation in HEK293T cells (Martens *et al*., 2005).

4.3.5 cAMP-mediated SOCS3-independent Inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 Activation in AS-Ms

With regards to AS-Ms, depletion of SOCS3 in these cells demonstrated no involvement of SOCS3 in the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 and ERK1,2 activation. As mentioned previously, other inhibitory mechanism could be responsible for this inhibition, independent of SOCS3. For example, in addition to PTP1B and other SOCS members, SHP2 could possibly be involved. Previous work in the Palmer lab has shown that pre-treatment of

HUVECs with the tyrosine phosphatase inhibitor mpV does not alter the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 activation (Sands *et al*., 2006). This argues against a role for SHP2 and other protein tyrosine phosphatases in the cAMP-mediated inhibition observed in HUVECs, but since this experiment was not performed in AS-Ms, SHP2 involvement could still be a possibility. Indeed, studies have shown that expression of catalytically inactive dominant negative SHP2 mutants results in increased gp130, JAK and STAT3 phosphorylation as well as gene induction (Lehmann *et al.*, 2003; Symes *et al.*, 1997).

4.3.6 cAMP-mediated SOCS3-dependent Inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 Activation in MEFs

Further support for the involvement of SOCS3 in the cAMP-mediated inhibition of sIL-6Rα/IL-6 signalling in HUVECs was demonstrated when the inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation was shown to be severely attenuated in $SOCS3^{-/-}$ MEFs, while remaining intact in the $SOCS3^{+/+}$ MEFs. These results strengthen the involvement of SOCS3 in the inhibitory mechanism and also show that the SOCS3-mediated inhibitory effects of cAMP on IL-6Rα/IL-6 signalling were not limited to HUVECs and may be a feature common to various cell types. Indeed, previous work in the Palmer lab has shown that the cAMP/SOCS3 inhibitory pathway is also present in human aortic endothelial cells (HAECs), as observed by a cAMP-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 activation and SOCS3 induction in these cells (Sands *et al*., 2006).

4.3.7 PMA-stimulated ERK1,2 Activation in HUVECs – Insensitivity to cAMP Inhibitory Mechanism

Further weight to the cAMP/SOCS3 pathway was added when examining the PMA effects in HUVECs. PMA, a direct protein kinase C (PKC) activator is known to intercept the ERK1,2 pathway at the level of Raf-1 (Kolch *et al*., 1993). This is supported by the findings that ERK activation following phorbol ester treatment does not require functional p21Ras, since a virus expressing dominant negative Asn17 mutant p21Ras in Rat-1 cells inhibits PDGF-mediated ERK2 activation, but not PMA-mediated ERK2 activation (de Vries-Smits *et al*., 1992). Furthermore, activation of p21Ras following PMA treatment could not be demonstrated under conditions in which insulin-induced p21Ras activation was readily observed (Medema *et al*., 1991). However, there is also evidence to suggest a Ras-

dependent activation of ERK1,2 following PMA treatment. Interestingly, this Ras-dependent activation of ERK1,2 in PMA-treated bovine pulmonary artery endothelial cells (BPAECs) was found to lead to endothelial barrier dysfunction (Verin *et al*., 2000). Further to a Raf-1-dependent and a Ras-dependent PMA/PKC-mediated ERK1,2 activation, other diacylglycerol/phorbol ester effectors have been identified. These include chimaerins, protein kinase D, RasGRPs, Munc13s and DAG kinase γ (Brose & Rosenmund, 2002). Of particular interest are the RasGRP effectors, since these comprise a family of 4 guanine nucleotide exchange proteins (GEFs) for Ras, all of which activate Ras in response to DAG/phorbol ester stimulation independent of PKC (Brose & Rosenmund, 2002). SOCS3 intercepts the ERK1,2 pathway at the level of the receptor, upstream of Raf-1 and Ras, while the well documented PKA/Raf-1 inhibitory pathway, mentioned previously, intercepts the ERK1,2 pathway at the level of Raf-1. In the present study, a cAMP-mediated inhibition of ERK1,2 activation following PMA stimulation in HUVECs could not be demonstrated. These results therefore suggest that the cAMP-mediated inhibitory effect observed in the present study does not appear to be intercepting the ERK1,2 pathway at the level of Ras or Raf-1, since PMA-stimulated ERK1,2 activation is still intact. Thus, the well described PKA-mediated inhibitory mechanism at the level of Raf-1 may not be operating, making the cAMP/SOCS3 pathway a more likely inhibitory mechanism. Furthermore, cAMP-mediated inhibition of PMA-stimulated ERK1,2 activation could not be shown by pre-treating the cells with Fsk and Roli for shorter incubations. This is in contrast to other studies, which have demonstrated a cAMP-mediated inhibition of PMA-stimulated ERK1,2 activation by pre-treating cells with cAMP-elevating agents for 5 minutes in adipocytes and CHO cells (Bradley *et al*., 1993), 10 minutes in A14 cells (Burgering *et al*., 1993) and 15 minutes in MC3T3-E1 osteoblasts (Siddhanti *et al*., 1995). Thus, the short-term effects of cAMP on PMA-stimulated ERK1,2 activation still do not support a role for the PKA/Raf-1 pathway.

4.3.8 cAMP-induced ERK1,2 Activation in HUVECs

Interestingly, treating HUVECs with Fsk and Roli for a shorter length of time (15 minutes) demonstrated activation of ERK1,2, whereas longer incubations (5 hours) showed no ERK1,2 activation. Additionally, this cAMP-mediated ERK1,2 activation was shown to be PKA-dependent. Previous results from the Palmer lab have

demonstrated that cAMP-mediated induction of SOCS3 in HUVECs is abolished by pre-treating the cells with the MEK inhibitor U0126. Furthermore, a selective JAK inhibitor was shown to have no effect on cAMP-mediated SOCS3 induction, but abolished SOCS3 induction in response to sIL-6Rα/IL-6 (Sands *et al*., 2006). Recently, Yarwood *et al*. (2008) suggested that the family of transcription factors termed CCAATT/enhancer binding proteins (CEBPs) link Epac activation to SOCS3 induction (Yarwood *et al.*, 2008). These findings are in contrast to the cAMP-mediated PKA-dependent activation of ERK1,2 observed in the present study, but are consistent with the cAMP-mediated PKA-independent inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation. Thus, the PKA-mediated stimulation of ERK1,2 may be distinct from the PKA-independent inhibitory mechanism. Intriguingly, further experiments demonstrated that ERK1,2 activation appears to be required for the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 activation, since the MEK inhibitor U0126 severely impaired STAT3 inhibition. This result also suggests that the inhibitory effects of cAMP were not due to an inactivation of dual specificity ERK phosphatases, such as the aforementioned MKP-1. Of interest, ERK1,2 activation in response to Fsk and Roli could not be observed in AS-Ms. This could possibly explain earlier results wherein SOCS3 siRNA had no effect on the inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation in AS-Ms. However, results from Chapter 3 demonstrated SOCS3 induction in response to Fsk and Roli treatment in AS-Ms. Thus, although Fsk and Roli treatment triggers SOCS3 induction in these cells, the induction may be completely distinct from the cAMP-mediated inhibitory effects on sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 phosphorylation in AS-Ms. It is tempting to speculate that a strong stimulation of cAMP-mediated ERK1,2 activation is required to observe cAMP/SOCS3 inhibition of IL-6 signalling in AS-Ms.

4.3.9 cAMP-mediated Epac1-dependent Inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation in HUVECs

As no pharmacological inhibitors of Epac currently exist, Epac siRNA is a powerful tool to determine the role of Epac in various processes, such as the regulation of EC permeability and the induction of SOCS3 (Kooistra *et al.*, 2005; Sands *et al.*, 2006). In the present study, the depletion of Epac1 in HUVECs had the effect of

severely attenuating the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced ERK1,2 and STAT3 activation. These results strongly implicate Epac1 in the cAMP-mediated inhibitory mechanism, which is consistent with other studies demonstrating Epac-mediated inhibitory effects in HUVECs, namely reduced vascular permeability and enhanced vascular endothelial (VE) cadherin-mediated cell-cell interactions. These effects were proposed to occur *via* a cAMP/Epac/Rap1 pathway, which is independent of PKA (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005). These results are also consistent with the anti-inflammatory effects of cAMP in endothelial cells (Blease *et al.*, 1998; Morandini *et al.*, 1996; Pober *et al.*, 1993). Therefore, targeting the cAMP/Epac/Rap1 pathway could prove to be beneficial in terms of limiting a number of endothelial functions associated with endothelial dysfunction. In comparison, and as mentioned previously, findings from Sobota and co-workers (2008) demonstrated a PKA-dependent and Epac-independent inhibition of sIL-6Rα/IL-6-induced ERK1,2 activation in human dermal fibroblasts (Sobota *et al*., 2008). It is interesting to note that the SOCS-mediated inhibitory mechanism observed in the present study targets gp130 and thus both the sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 pathways in response to cAMP elevation, whereas the PKA-dependent inhibitory mechanism observed in Sobota *et al*. (2008) targets Raf-1 in response to cAMP elevation and thus only the sIL-6Rα/IL-6-stimulated ERK1,2 pathway.

4.3.10 cAMP Selectivity - Compartmentalisation

How and why cAMP selectively activates some pathways in some cells and other pathways in other cells is still unclear. This is particularly exemplified in the selective activation of ERK1,2 in some cells and the inhibition of ERK1,2 in other cells in response to cAMP as discussed earlier in this Chapter. This may be due to a number of reasons. For example, the signalling proteins that couple different pathways may be expressed in a cell type dependent manner. Alternatively, and of interest to the present study there is increasing evidence to support the compartmentalization of cAMP within the cell, which may only permit the selective activation of certain signaling pathways.

Early models proposed that cAMP was distributed uniformly within cells, but it is now known that cAMP can also localise to discreet cellular compartments (Dodge-Kafka *et al.*, 2005; Netherton *et al.*, 2007). This is in part achieved by the

association of PKA with A-kinase anchoring proteins (AKAPs). AKAPs are non-enzymatic scaffolding proteins, which anchor PKA to specific subcellular structures (Michel & Scott, 2002). One of the first AKAPs to be described was AKAP75, originally identified as a contaminant in PKA type II preparations (Sarkar *et al*., 1984). Since then, the family of AKAPs has increased to over 50 members. Of relevance to the present study, muscle-specific mAKAP signalling complexes identified in the heart, have been shown to comprise PKA, Epac1, PDE4D3 and ERK5. As mentioned previously in section 1.2.2 of the Introduction, this complex incorporates 2 cAMP-mediated pathways and 2 coupled cAMP-dependent feedback loops. Briefly, both PKA and Epac1 can become activated *via* ERK phosphorylation of PDE4D3 on Ser579 (Hoffmann *et al*., 1999), which suppresses phosphodiesterase activity leading to increased cAMP levels. Activated PKA can then phosphorylate PDE4D3 on Ser54 (Sette & Conti, 1996), which increases its affinity for cAMP and then decreases localised cAMP levels. Additionally, activated Epac1 can inhibit ERK5, thus preventing continued inactivation of PDE4D3 (Dodge-Kafka *et al*., 2005) (Figure 4.1). This is in contrast to other studies as Epac- and PKA-integrated signalling is observed within the same signalling complex, whereas other studies have shown that PKA- and Epac-based complexes are distinct and cannot be found together (Netherton *et al.*, 2007; Raymond *et al.*, 2007). Furthermore, these conflicting studies also demonstrate that PDE3B and PDE4D are similarly non-overlapping, with PKA- or Epac-based signalling complexes containing either PDE3B or PDE4D but not both (Netherton *et al.*, 2007; Raymond *et al.*, 2007). Raymond and coworkers (2007) proposed that at least 3 signalling complexes were present in 293T cells; PDE3B-EPAC, PDE4D-EPAC and PDE4D-AKAP-PKA (Raymond *et al*., 2007). Of interest, further work by this group showed that the PDE3B-EPAC complex was also found in human aortic endothelial cells (HAECs) (Netherton *et al*., 2007). When examining the mAKAP signalling complex in rat neonatal ventriculocytes (RNVs), pre-treatment with forskolin for 20 minutes resulted in a reduction in serum-dependent activation of mAKAP-associated ERK5, which could not be rescued by the PKA inhibitor H89 (Dodge-Kafka *et al*., 2005). Similar results were observed when using alternative PKA inhibitors, such as KT5720 and Rp-cAMPs, thereby implicating a PKA-independent mechanism of cAMP–mediated inhibition of ERK5 (Dodge-Kafka *et al*., 2005). 8pCPT was used to assess whether Epac1 was

playing a role in mAKAP-associated ERK5 inhibition, and results showed that 8pCPT treatment for 1hr recapitulated the inhibitory effect of forskolin on serum-dependent activation of ERK5, thereby suggesting the involvement of Epac1, and not PKA, in mAKAP-associated ERK5 inhibition (Dodge-Kafka *et al*., 2005). Furthermore, the same group implicated Rap-1 in the inhibitory effect, since expression of a constitutively active RapGAP blocked cAMP–mediated inhibition of ERK5 (Dodge-Kafka *et al*., 2005). These results parallel the present results to a degree and may possibly suggest the presence of an analogous signalling complex in the present study.

4.3.11 Possible Downstream Effects of ERK1,2 Activation

Although the cAMP-mediated inhibitory mechanism was the main focus of the present study, it should be noted that in an attempt to investigate this inhibitory effect on ERK1,2 signalling further downstream, the transcriptional activity of the well characterised ERK-responsive transcription factor Elk-1 was shown to be unaffected by sIL-6Rα/IL-6 or leptin treatment in HUVECs (and AS-Ms). However, a trend of increased Elk-1 transcriptional activity following PMA treatment was observed in HUVECs (and AS-Ms). These findings were largely supported by the results from the nuclear extraction experiments, as ERK1,2 activation following sIL-6Rα/IL-6- or leptin treatment in the nuclear fractions of both HUVECs and AS-Ms was substantially lower when compared to PMA-induced ERK1,2 activation in the nuclear fractions of both cell types. A possible explanation for these observations may involve the spatial regulation of MAPK and in particular the nucleocytoplasmic trafficking of ERK1,2. Sef (similar expression to fgf genes) is a transmembrane protein which has been shown to sequester activated MEK1,2/ERK1,2 complexes in the cytoplasm by preventing MEK1,2 dissociation. MEK1,2 dissociation is required for ERK1,2 translocation to the nucleus. Thus, Sef blocks ERK1,2 translocation, and does not appear to affect the activity of ERK1,2 in the cytoplasm (Kondoh *et al*., 2005). Of relevance to the present study, Elk-1 transcriptional activity in response to FGF and EGF treatment in HEK293 cells and HeLa cells was shown to be inhibited following transfection with human Sef (hSef) constructs. In comparison, activation of p90 ribosomal S6 kinase 2 (RSK2) by FGF, a well known cytoplasmic ERK1,2 substrate was unaffected by hSef (Torii *et al*., 2004). In addition to Sef, another protein which has been shown to influence

ERK1,2 localisation is phosphoprotein enriched in astrocytes 15 kDa (PEA-15). PEA-15 has been shown to promote cytoplasmic localisation of ERK1,2 by binding to ERK1,2 and RSK2 and sequestering them both in the cytoplasm. PEA-15 is believed to act as a scaffold protein, enhancing ERK1,2 phosphorylation of RSK2. Lymphocytes from PEA-15 knock-out mice demonstrated diminished RSK2 activity in response to PMA, which is rescued by exogenous PEA-15 expression (Vaidyanathan *et al*., 2007). Thus, the possible involvement of Sef and/or PEA-15 in sIL-6Rα/IL-6- or leptin-induced ERK1,2 activation could result in the preferential activation of cytoplasmic substrates, as opposed to nuclear substrates. Indeed, a study conducted in human bone marrow stromal cells showed that leptin induced apoptosis through the ERK1/2 cascade *via* activation of cytosolic phospholipase A2 (cPLA2) (Kim *et al*., 2003) In comparison to Sef and PEA-15 which both act to sequester ERK1,2 in the cytoplasm, the kinase suppressor of Ras (KRS) has been shown to inhibit Elk1 activity by promoting Elk-1 dephosphorylation *via* activation of the major Elk-1 phosphatase; phosphoprotein phosphatase 2B (PP2B, also known as calcineurin). EGF- and Ras-induced activation of Elk-1 in COS1 cells has been shown to be inhibited by KSR, whilst ERK1,2 activation is unaffected (Sugimoto *et al*., 1998). The involvement of KSR could possibly explain the lack of PMA-induced Elk-1 transcriptional activity despite observing a marked increase in ERK1,2 activation in the nuclear fractions following PMA treatment. However, further to the possible regulation of ERK1,2 and /or Elk-1 outlined above, consideration must also be given to experimental drawbacks such as low transfection efficiency.

4.3.12 Mechanism of Inhibition - Summary

In summary, and in relation to the inhibitory mechanism, results from this Chapter demonstrate a cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation in HUVECs, which is independent of PKA. This cAMP-mediated inhibition was also shown to be Epac1- and SOCS3-dependent. Further to this, there appeared to be a requirement for ERK1,2 activation in the cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated STAT3 activation in HUVECs. In contrast to these findings, cAMP-mediated inhibition of leptin-stimulated STAT3 activation in HUVECs was shown to occur *via* a SOCS3- independent mechanism. The responses to cAMP elevation on sIL-6Rα/IL-6- and leptin-stimulated ERK1,2

activation in AS-Ms were variable, since basal levels of ERK1,2 activation were high. Moreover, the responses to cAMP elevation on sIL-6Rα/IL-6- and leptin-stimulated STAT3 activation were either very modest or showed no effect, respectively. However, siRNA experiments demonstrated that SOCS3 was not involved in the cAMP-mediated inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation in AS-Ms. Focusing on the results relevant to the working hypothesis of this study, the present results together with previous results from the Palmer lab and Yarwood *et al*. (2008) show an Epac1-mediated accumulation of GTP-bound Rap1a which is sufficient to induce SOCS3 expression possibly *via* the C/EBP family of transcription factors. SOCS3 accumulation subsequently leads to the inhibition of sIL-6Rα/IL-6-stimulated ERK1,2 and STAT3 activation in HUVECs. These inhibitory effects appear to be both ERK1,2-dependent and PKA-independent.

Figure 4.1 mAKAP Complex in Cardiomyocytes

Cytokines such as LIF activate ERK5, which phosphorylates PDE4D3 on Ser579, causing its inactivation. This leads to an increase in local cAMP levels and the subsequent activation of PKA and Epac1. Activated PKA phosphorylates PDE4D3 on Ser54, which increases its affinity for cAMP and lowers the levels of cAMP. Additionally, activated Epac1 inhibits ERK5 *via* Rap1, thus preventing continued inactivation of PDE4D3. Therefore, both PKA and Epac1 mediate negative feedback of local cAMP levels within this multi-protein complex.
(Taken from (Bos, 2006).

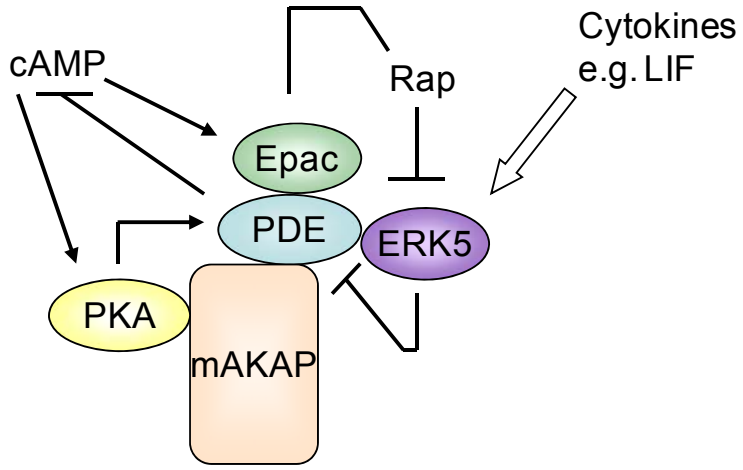
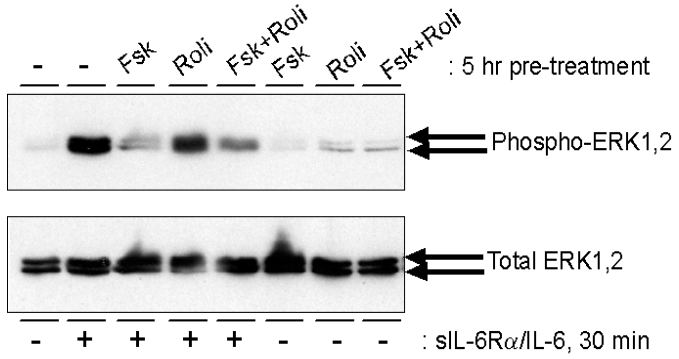


Figure 4.2 The Effect of cAMP elevating agents on ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively, and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells, ***p<0.001 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk+Roli Fsk Roli Fsk+Roli : 5 hr pre-treatment
Phospho-ERK1,2
Total ERK1,2
- + + + + - - - : sIL-6Rα/IL-6, 30 min

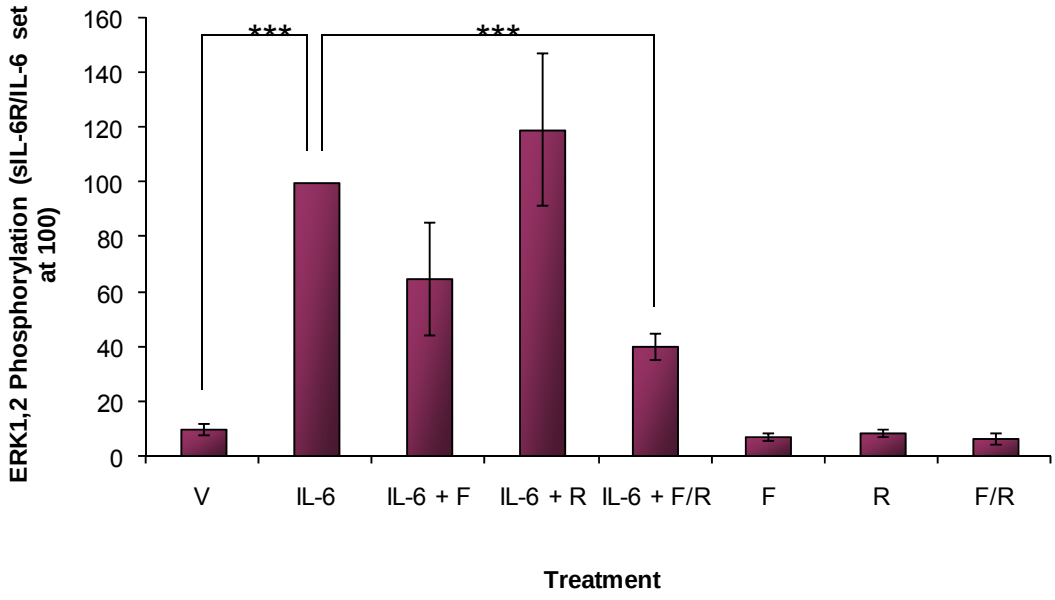
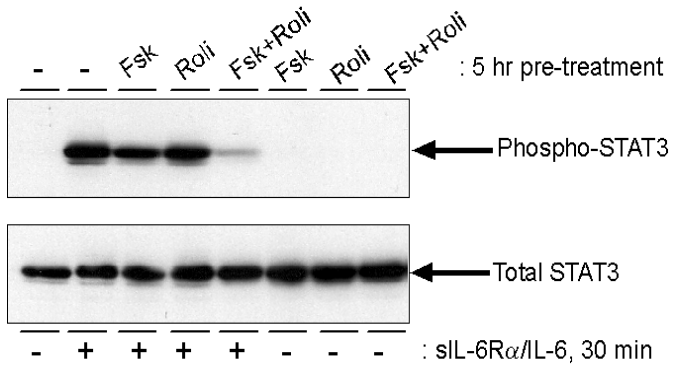

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
160
140
120
100
80
60
40
20
0
***
***
V
IL-6
IL-6 + F
IL-6 + R
IL-6 + F/R
F
R
F/R
Treatment

Figure 4.3 The Effect of cAMP elevating agents on STAT3 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (****p*<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, ****p*<0.001 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk+Roli Fsk Roli Fsk+Roli : 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + + + - - - : sIL-6Rα/IL-6, 30 min

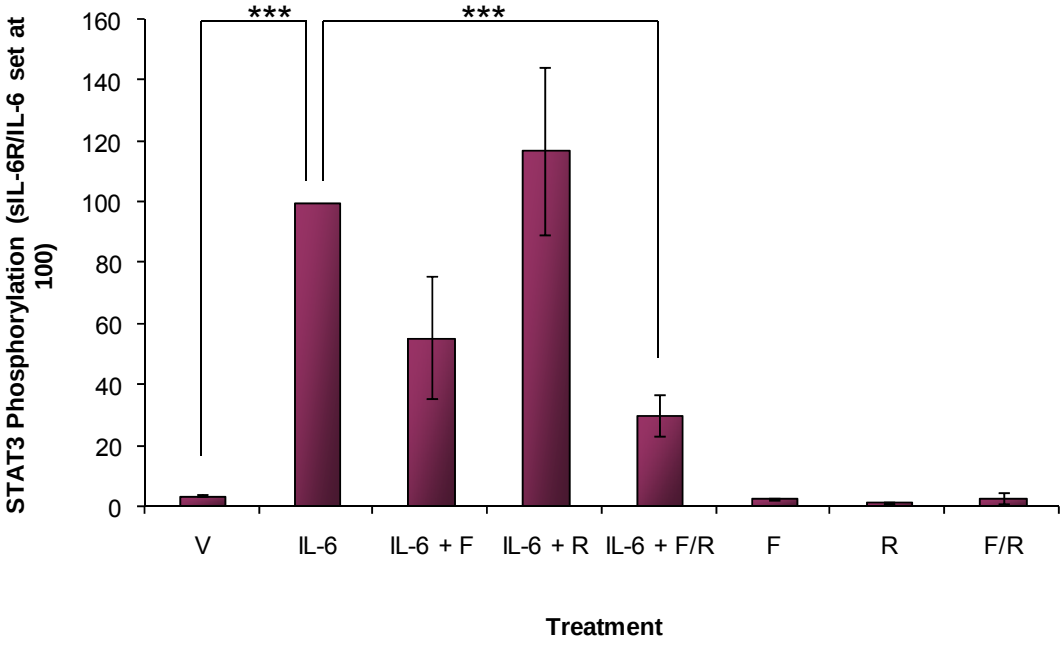
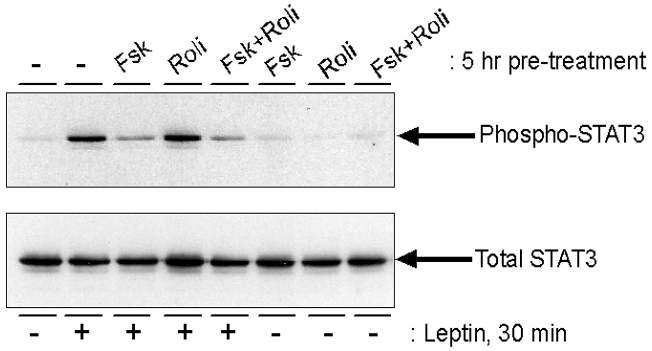

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
160
140
120
100
80
60
40
20
0
***
***
V
IL-6
IL-6 + F
IL-6 + R
IL-6 + F/R
F
R
F/R
Treatment

Figure 4.4 The Effect of cAMP elevating agents on STAT3 Phosphorylation by Leptin in HUVECs

4×10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without leptin at a concentration of 125ng/ml for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and serum-free medium at the same volume and for the same length of time as Fsk, Roli and leptin treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, **p<0.01 / *p<0.05 *versus* phosphorylated STAT3 levels in leptin treated alone cells). Leptin-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk+Roli Fsk Roli Fsk+Roli
: 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + + + - - -
: Leptin, 30 min

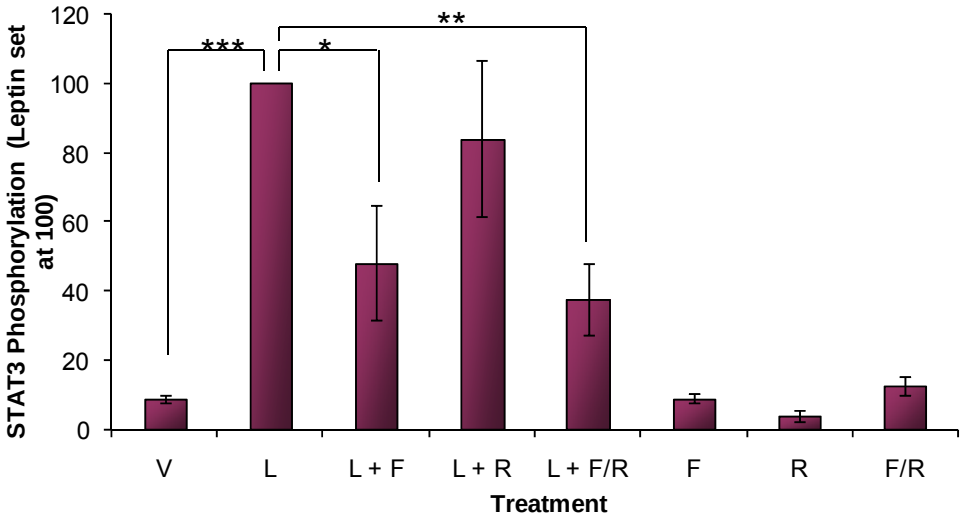
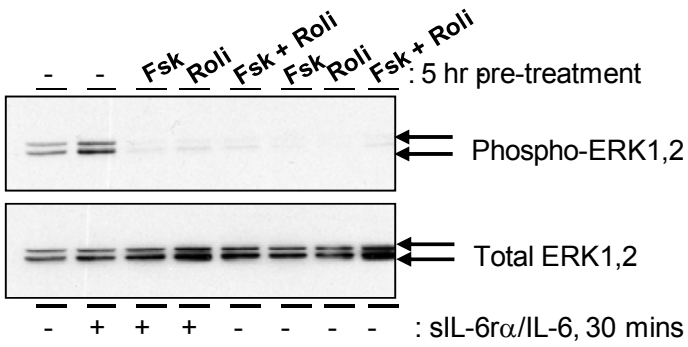

STAT3 Phosphorylation (Leptin set at 100)
120
100
80
60
40
20
0
***
*
**
V
L
L + F
L + R
L + F/R
F
R
F/R
Treatment

Figure 4.5 The Effect of cAMP elevating agents on ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively, and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk + Roli Fsk Roli Fsk + Roli : 5 hr pre-treatment
Phospho-ERK1,2
Total ERK1,2
- + + + - - - - : sIL-6rα/IL-6, 30 mins

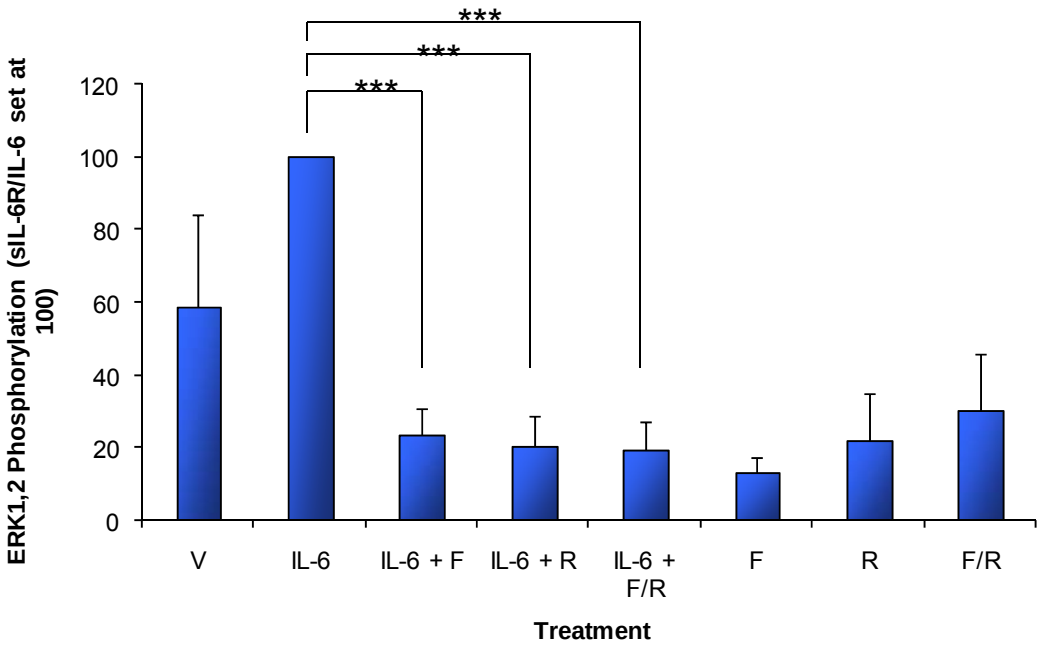
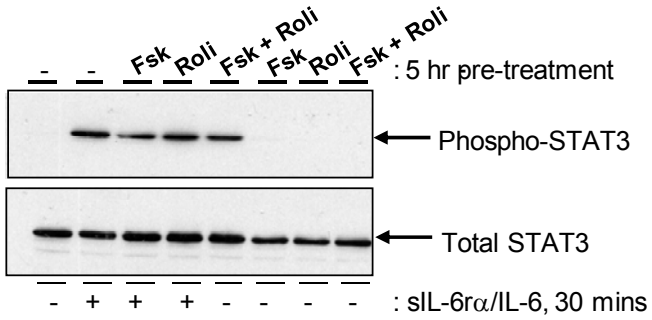

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
***
V
IL-6
IL-6 + F
IL-6 + R
IL-6 + F/R
F
R
F/R
Treatment

Figure 4.6 The Effect of cAMP elevating agents on STAT3 Phosphorylation by sIL-6Rα/IL-6 in AS-Ms

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p*<0.01 *versus* phosphorylated STAT3 levels in vehicle-treated cells, **p*<0.05 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk + Roli Fsk Roli Fsk + Roli
: 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + + - - - - : sIL-6rα/IL-6, 30 mins

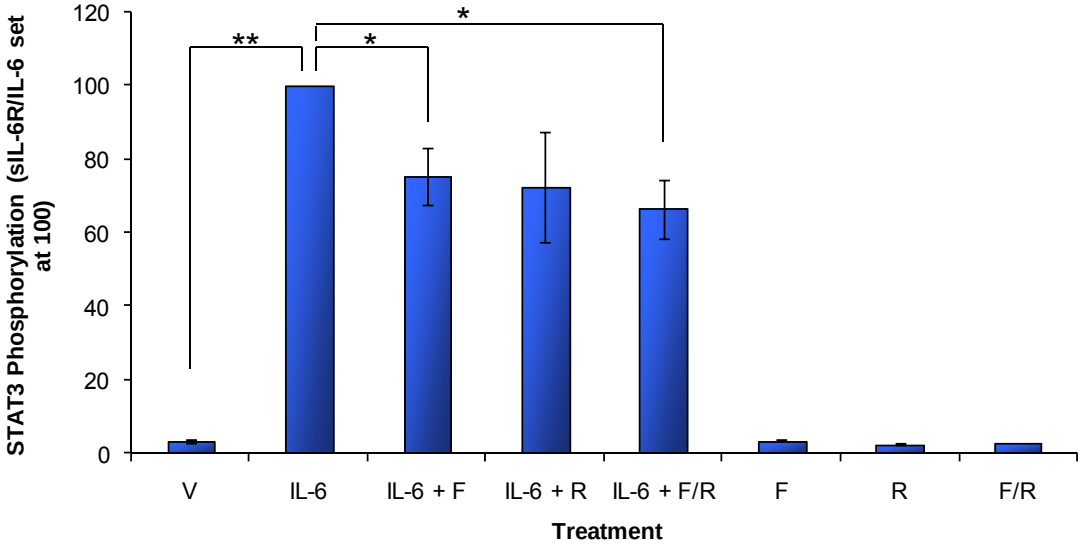
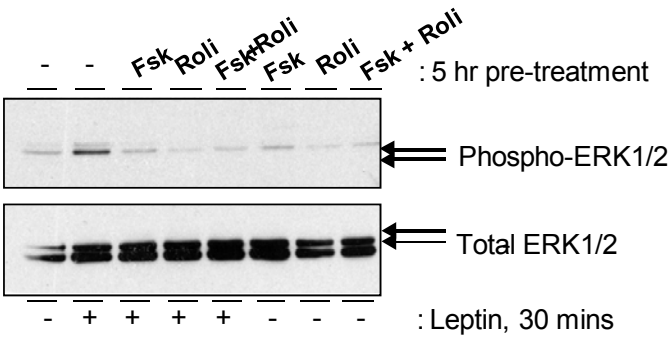

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
**
*
*
V
IL-6
IL-6 + F
IL-6 + R
IL-6 + F/R
F
R
F/R
Treatment

Figure 4.7 The Effect of cAMP elevating agents on ERK1,2 Phosphorylation by Leptin in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without leptin at a concentration of 125ng/ml for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and serum-free medium at the same volume and for the same length of time as Fsk, Roli and leptin treatment respectively, and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated ERK1,2 levels in leptin-treated alone cells). Leptin-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk+Roli Fsk Roli Fsk + Roli
: 5 hr pre-treatment
Phospho-ERK1/2
Total ERK1/2
- + + + + - - -
: Leptin, 30 mins

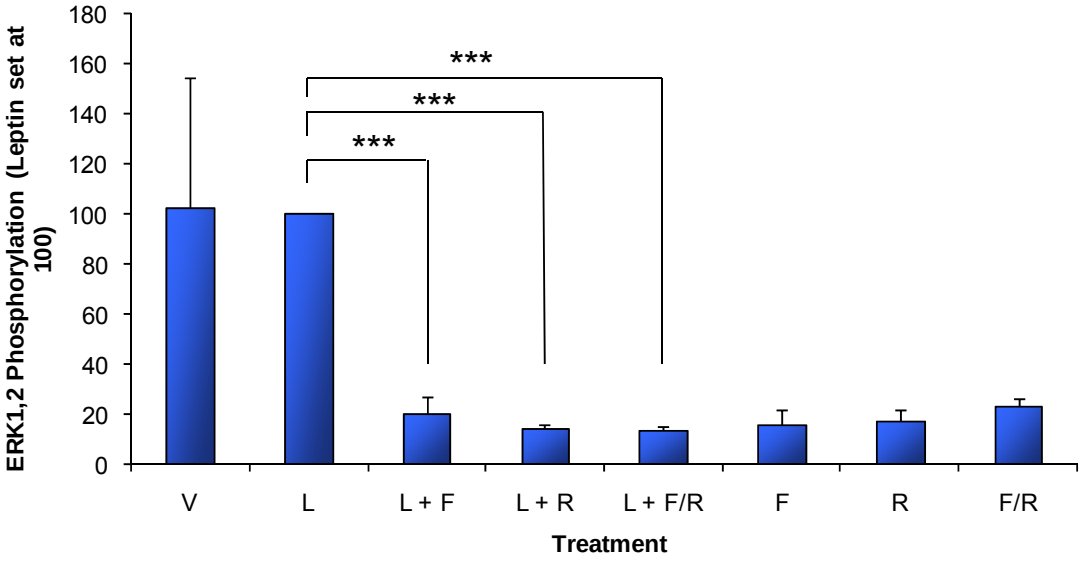
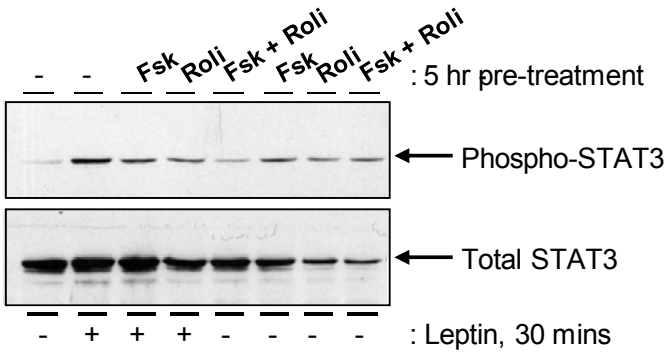

ERK1,2 Phosphorylation (Leptin set at 100)
180
160
140
120
100
80
60
40
20
0
***
***
***
V
L
L + F
L + R
L + F/R
F
R
F/R
Treatment

Figure 4.8 The Effect of cAMP elevating agents on STAT3 Phosphorylation by Leptin in AS-Ms

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and/or 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without leptin at a concentration of 125ng/ml for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and serum-free medium at the same volume and for the same length of time as Fsk, Roli and leptin treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells). Leptin-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk Roli Fsk + Roli Fsk Roli Fsk + Roli
: 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + + - - - -
: Leptin, 30 mins

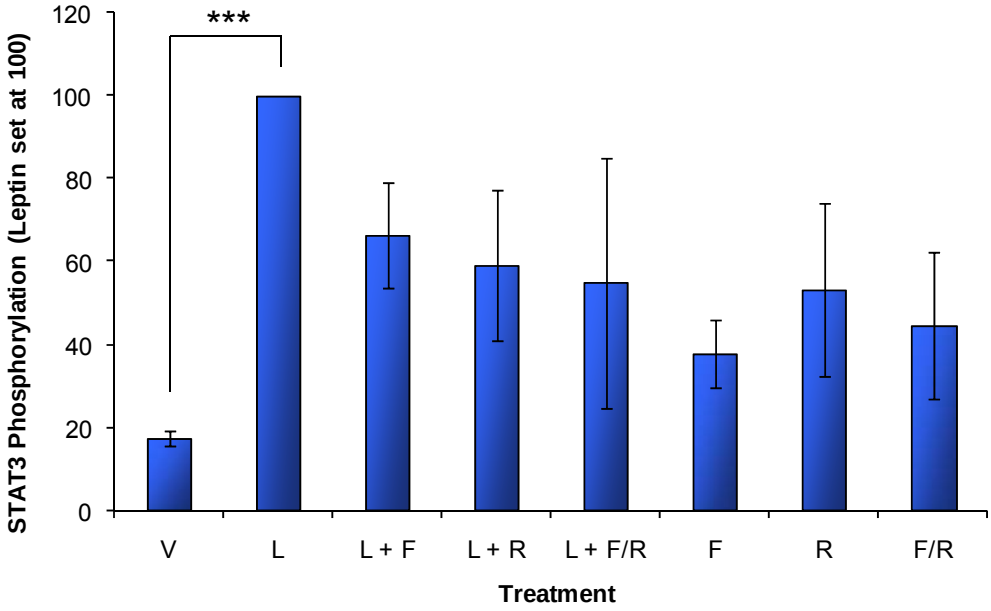

***
120
100
80
60
40
20
0
STAT3 Phosphorylation (Leptin set at 100)
V
L
L + F
L + R
L + F/R
F
R
F/R
Treatment

Figure 4.9 The Effect of sIL-6Rα/IL-6 and Leptin Treatment on the Transcriptional Activity of Elk1 in HUVECs and AS-Ms

3 x 10^5 HUVECs/well and 3.5 x 10^5 AS-Ms/well were seeded into 6 well plates. 24 hrs after seeding, medium was replaced with fresh medium and the cells were transfected with a *trans*-activator plasmid (Gal4-Elk-1) at 1µg/well, a luciferase reporter plasmid (Gal4-luc) at 1µg/well and a normalisation Renilla plasmid (pRL-CMV) at 0.5µg/well. Cells were incubated overnight before another change of medium and left for a further 24 hrs. Following 24 hrs, cells were treated with or without 1µM U0126 for 30 minutes prior to treatment with sIL-6Rα/IL-6 (25ng/ml/5ng/ml) or leptin (125ng/ml) for 6 hours. The controls in this experiment included vehicle (V) treated cells, which were transfected with all three constructs and 1µM PMA treated cells, a potent activator of ERK1,2 in endothelial cells. Following treatment, cell lysates were assayed for Firefly and *Renilla* luciferase activity, using a luminometer. The results are expressed as fold increase +/- standard error from 3 independent experiments performed in triplicate in HUVECs (a.) and AS-Ms (b.). Graph (c) shows luciferase activity from one biological replicate assayed in triplicate in HUVECs (**p*<0.05 *versus* luciferase activity in vehicle-treated cells). [U1=U0126].

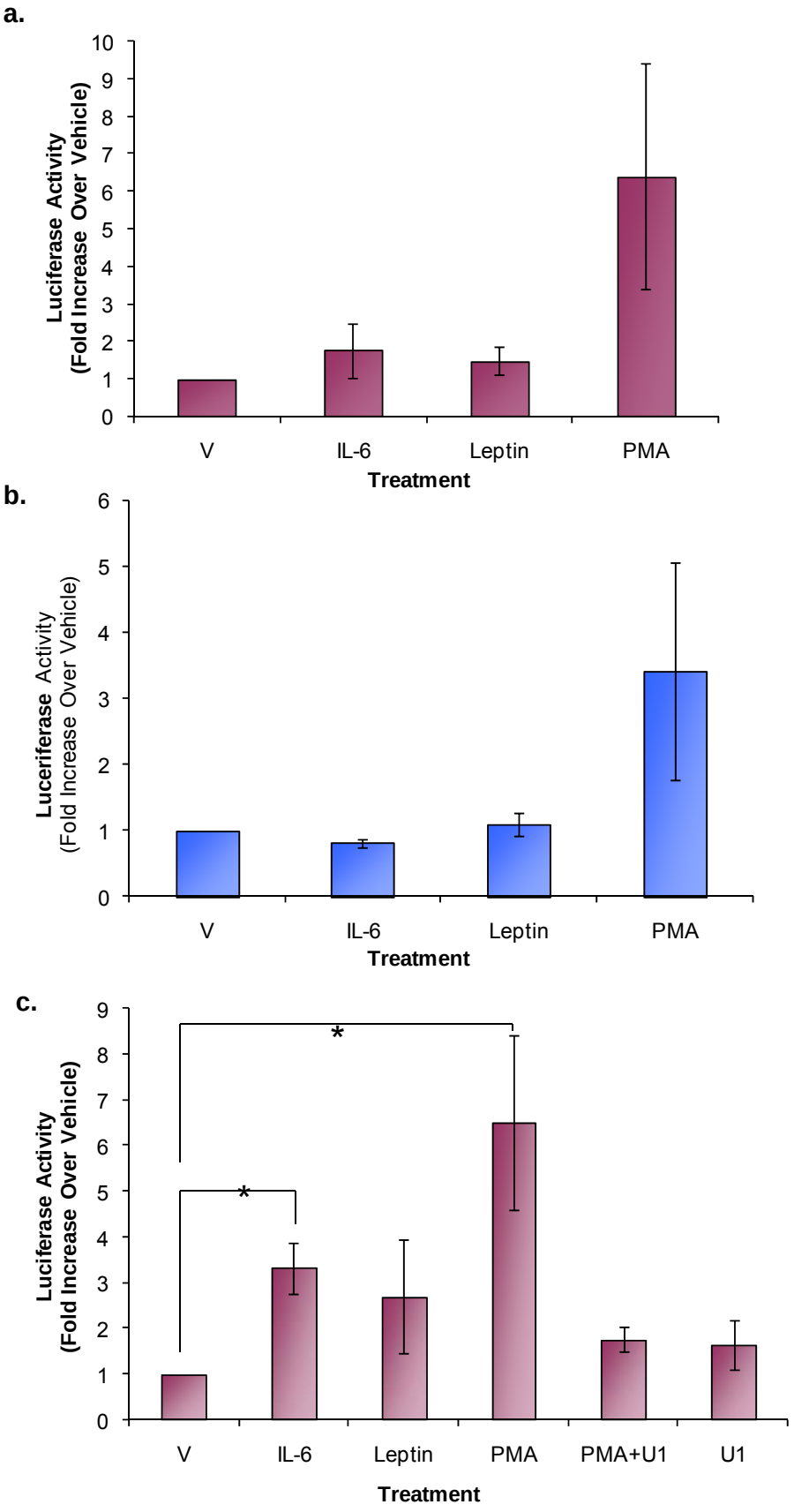

a.
Luciferase Activity
(Fold Increase Over Vehicle)
10
9
8
7
6
5
4
3
2
1
0
V
IL-6
Leptin
PMA
Treatment
b.
Luceriferase Activity
(Fold Increase Over Vehicle)
6
5
4
3
2
1
0
V
IL-6
Leptin
PMA
Treatment
c.
Luciferase Activity
(Fold Increase Over Vehicle)
9
8
7
6
5
4
3
2
1
0
*
*
V
IL-6
Leptin
PMA
PMA+U1
U1
Treatment

Figure 4.10 The Effect of sIL-6Rα/IL-6 and Leptin Treatment on ERK1,2 and STAT3 Activation and Translocation to the Nucleus in HUVECs

$12x10^5$ HUVECs were seeded into $10cm^2$ dishes. 24 hrs after seeding, cells were washed twice in PBS and serum starved for 4 hrs. Following 4 hrs, cells were treated with or without leptin (125ng/ml) or sIL-6Rα/IL-6 (25ng/ml/5ng/ml) for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with PBS and serum-free medium at the same volume and for the same length of time as sIL-6Rα/IL-6 and leptin treatment respectively and 1μM PMA, a potent activator of ERK1,2 in endothelial cells. Following treatment, cells were harvested in PBS. A series of centrifugation and washing steps were then undertaken, resulting in the isolation of nuclear and cytoplasmic cell fractions. Both fractions were equalised for protein concentration and fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. SP1, a transcription factor and tubulin, a cytoskeleton protein, were both analysed to confirm the isolation of nuclear and cytosolic preparations respectively. Quantitative analysis of phosphorylated ERK1,2 and STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001, *p<0.05 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells and **p<0.01 *versus* phosphorylated STAT3 in vehicle-treated cells). Basal is set at 100.

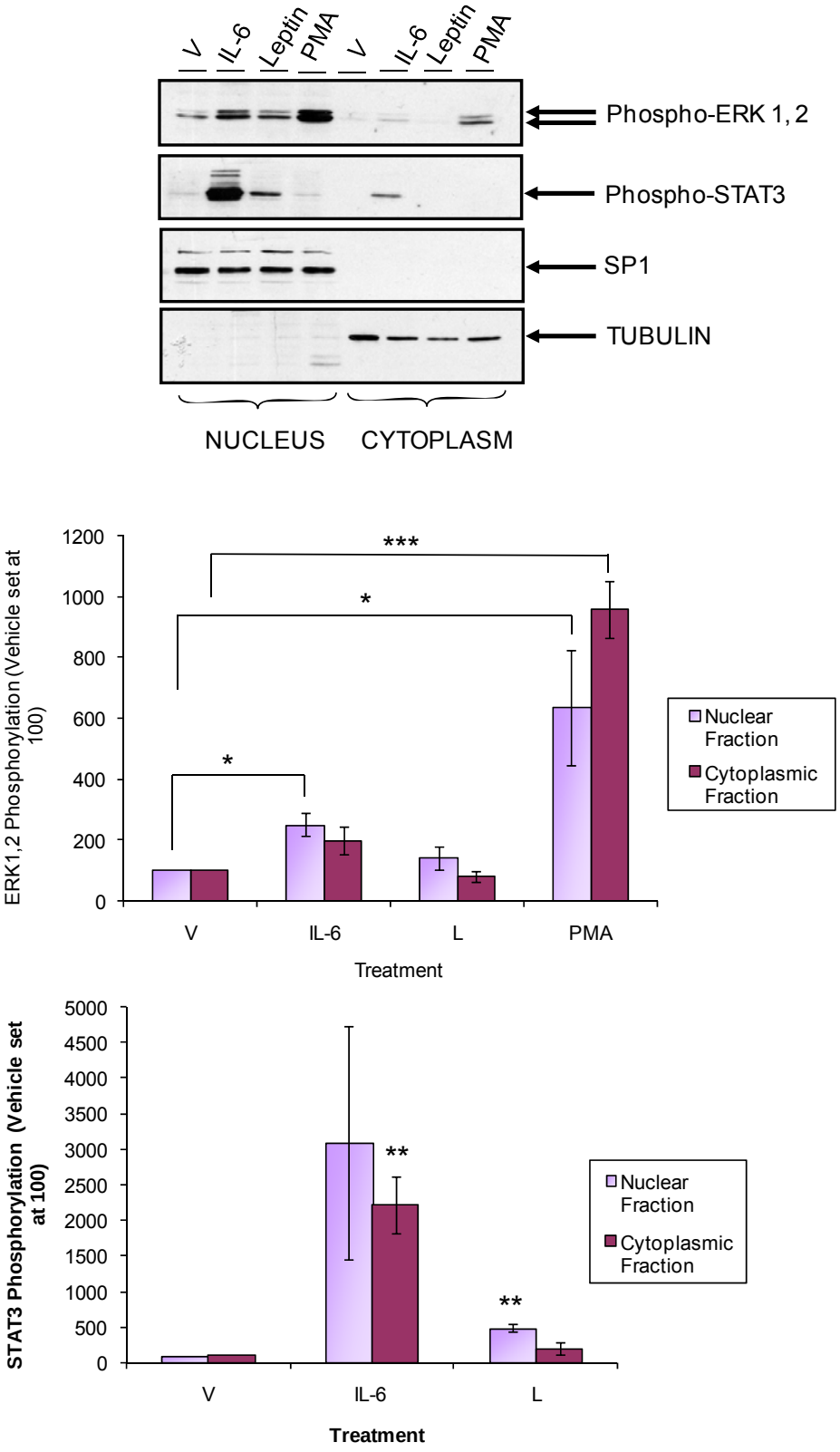
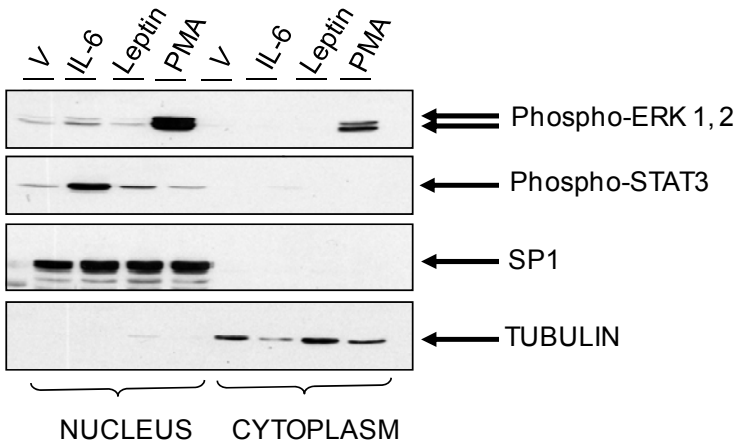
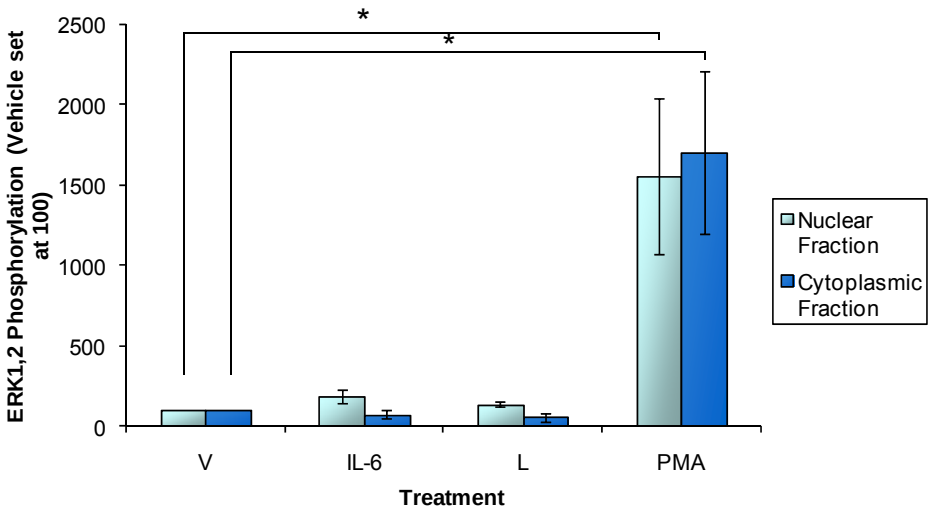

V
IL-6
Leptin
PMA
V
IL-6
Leptin
PMA
Phospho-ERK 1, 2
Phospho-STAT3
SP1
TUBULIN
NUCLEUS
CYTOPLASM
ERK1,2 Phosphorylation (Vehicle set at 100)
1200
1000
800
600
400
200
0
***
*
*
V
IL-6
L
PMA
Treatment
Nuclear Fraction
Cytoplasmic Fraction
STAT3 Phosphorylation (Vehicle set at 100)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
**
V
IL-6
L
Treatment
Nuclear Fraction
Cytoplasmic Fraction

Figure 4.11 The Effect of sIL-6Rα/IL-6 and Leptin Treatment on ERK1,2 and STAT3 Translocation to the Nucleus in AS-Ms

$12x10^5$ AS-Ms were seeded into $10cm^2$ dishes. 24 hrs after seeding, cells were washed twice in PBS and serum starved for 4 hrs. Following 4 hrs, cells were treated with or without leptin (125ng/ml) or sIL-6Rα/IL-6 (25ng/ml/5ng/ml) for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with PBS and serum-free medium at the same volume and for the same length of time as sIL-6Rα/IL-6 and leptin treatment respectively and 1μM PMA, a potent activator of ERK1,2 in endothelial cells. Following treatment, cells were harvested in PBS. A series of centrifugation and washing steps were then undertaken, resulting in the isolation of nuclear and cytoplasmic cell fractions. Both fractions preparations were equalised for protein concentration and fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. SP1, a transcription factor and tubulin, a cytoskeleton protein, were both analysed to confirm the isolation of nuclear and cytosolic preparations respectively. Quantitative analysis of phosphorylated ERK1,2 and STAT3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p*<0.01, **p*<0.05 *versus* phosphorylated ERK1,2 and phosphorylated STAT3 levels in vehicle-treated cells). Basal is set at 100.


V
IL-6
Leptin
PMA
V
IL-6
Leptin
PMA
Phospho-ERK 1, 2
Phospho-STAT3
SP1
TUBULIN
NUCLEUS
CYTOPLASM


ERK1,2 Phosphorylation (Vehicle set at 100)
2500
2000
1500
1000
500
0
*
*
V
IL-6
L
PMA
Treatment
Nuclear Fraction
Cytoplasmic Fraction

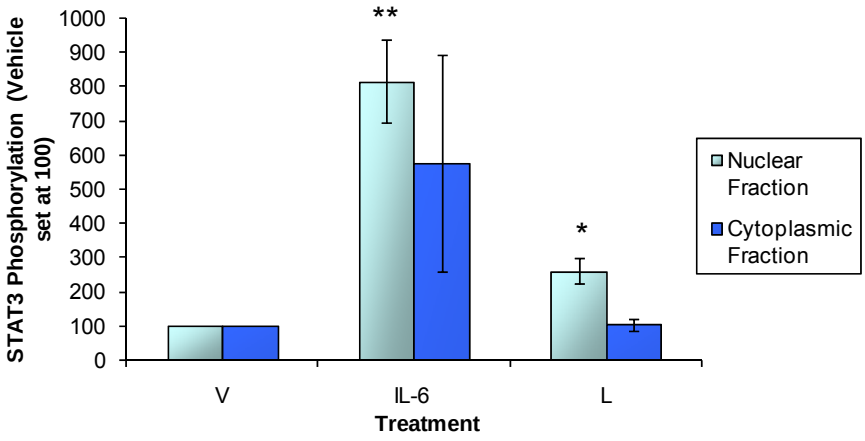
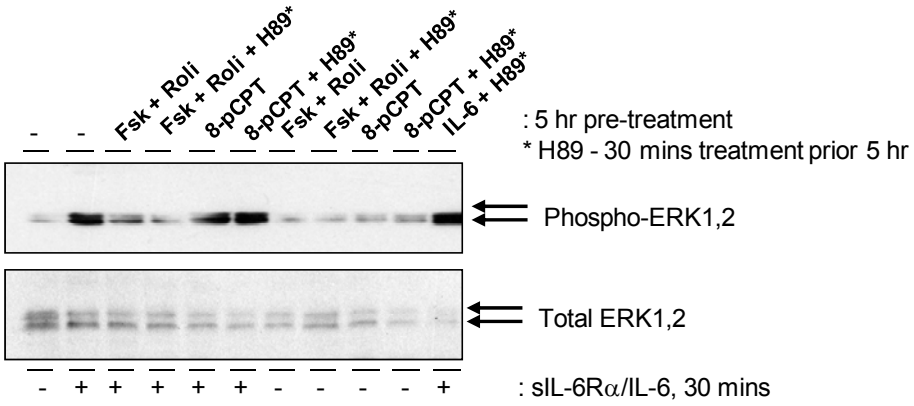

STAT3 Phosphorylation (Vehicle set at 100)
1000
900
800
700
600
500
400
300
200
100
0
**
*
V
IL-6
L
Treatment
Nuclear Fraction
Cytoplasmic Fraction

Figure 4.12 The Effect of H89 on cAMP-mediated Inhibition of ERK1,2 Phosphorylation and the Effect of 8pCPT on ERK Phosphorylation by sIL-6Rα/IL-6 in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 5μM H89 prior to treatment with 10μM forskolin (Fsk) and 10μM rolipram (Roli) or 100μM 8pCPT for 5 hrs in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively, and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p*<0.01 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells, ***p*<0.01 / **p*<0.05 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli, 8MC=8pCPT].


- - Fsk + Roli
Fsk + Roli + H89*
8-pCPT
8-pCPT + H89*
Fsk + Roli
Fsk + Roli + H89*
8-pCPT
8-pCPT + H89*
IL-6 + H89*
: 5 hr pre-treatment
* H89 - 30 mins treatment prior 5 hr
Phospho-ERK1,2
Total ERK1,2
- + + + + + - - - - +
: sIL-6Rα/IL-6, 30 mins

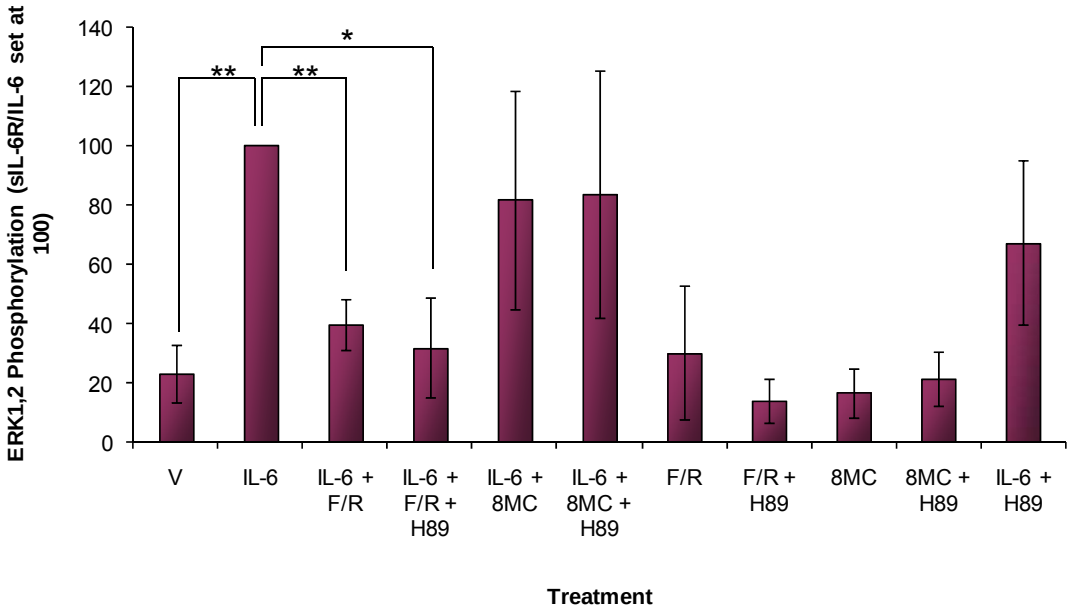
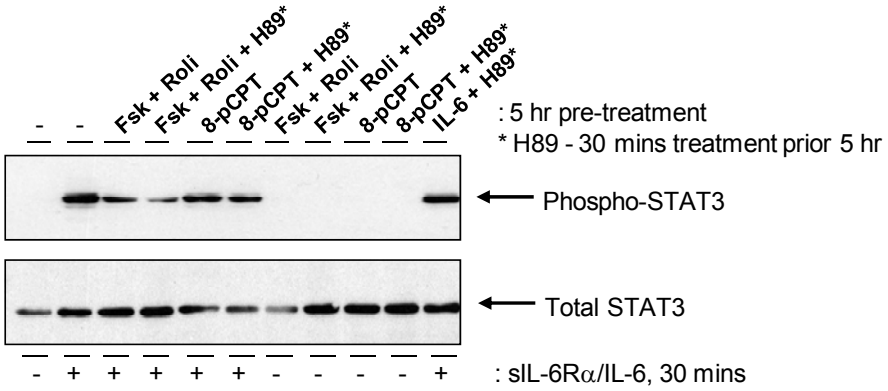

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
140
120
100
80
60
40
20
0
**
**
*
V
IL-6
IL-6 + F/R
IL-6 + F/R + H89
IL-6 + 8MC
IL-6 + 8MC + H89
F/R
F/R + H89
8MC
8MC + H89
IL-6 + H89
Treatment

Figure 4.13 The Effect of H89 on cAMP-mediated Inhibition of STAT3 Phosphorylation and the Effect of 8pCPT on STAT3 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

4 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 5μM H89 prior to treatment with 10μM forskolin (Fsk) and 10μM rolipram (Roli) or 100μM 8pCPT for 5 hrs in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, ***p<0.001 / *p<0.05 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli, 8MC=8pCPT].


- - Fsk + Roli
Fsk + Roli + H89*
8-pCPT
8-pCPT + H89*
Fsk + Roli
Fsk + Roli + H89*
8-pCPT
8-pCPT + H89*
IL-6 + H89*
: 5 hr pre-treatment
* H89 - 30 mins treatment prior 5 hr
Phospho-STAT3
Total STAT3
- + + + + + - - - - +
: sIL-6Rα/IL-6, 30 mins

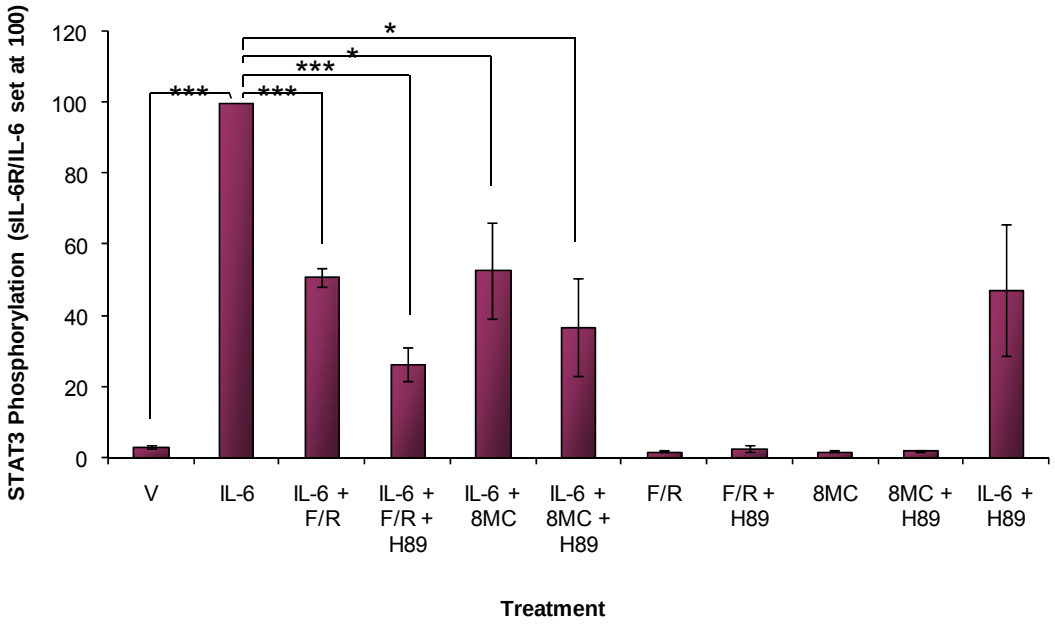
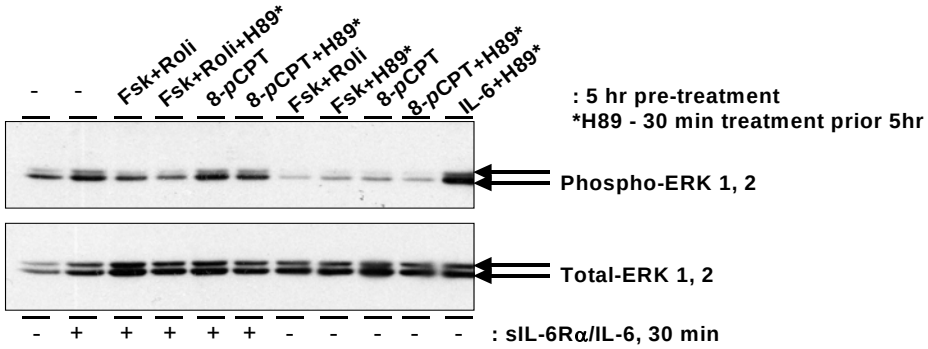

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
***
*
*
V
IL-6
IL-6 + F/R
IL-6 + F/R + H89
IL-6 + 8MC
IL-6 + 8MC + H89
F/R
F/R + H89
8MC
8MC + H89
IL-6 + H89
Treatment

Figure 4.14 The Effect of H89 on cAMP-mediated Inhibition of ERK1,2 Phosphorylation and the Effect of 8pCPT on ERK Phosphorylation by sIL-6Rα/IL-6 in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 5μM H89 prior to treatment with 10μM forskolin (Fsk) and 10μM rolipram (Roli) or 100μM 8pCPT for 5 hrs in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively, and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (**p<0.01 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli, 8MC=8pCPT].


- - Fsk+Roli Fsk+Roli+H89* 8-pCPT 8-pCPT+H89* Fsk+Roli Fsk+H89* 8-pCPT 8-pCPT+H89* IL-6+H89*
: 5 hr pre-treatment
*H89 - 30 min treatment prior 5hr
Phospho-ERK 1, 2
Total-ERK 1, 2
- + + + + + - - - - - : sIL-6Rα/IL-6, 30 min

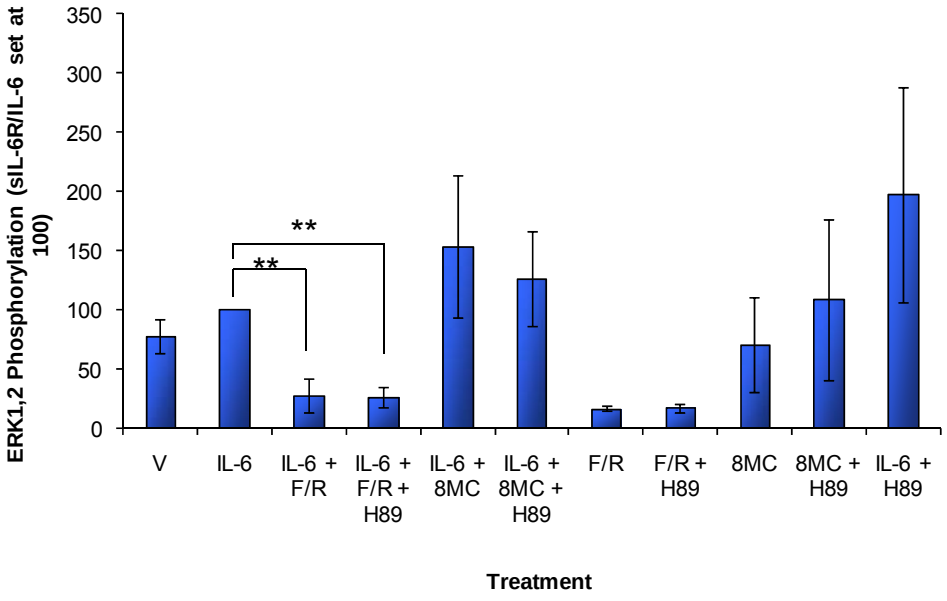
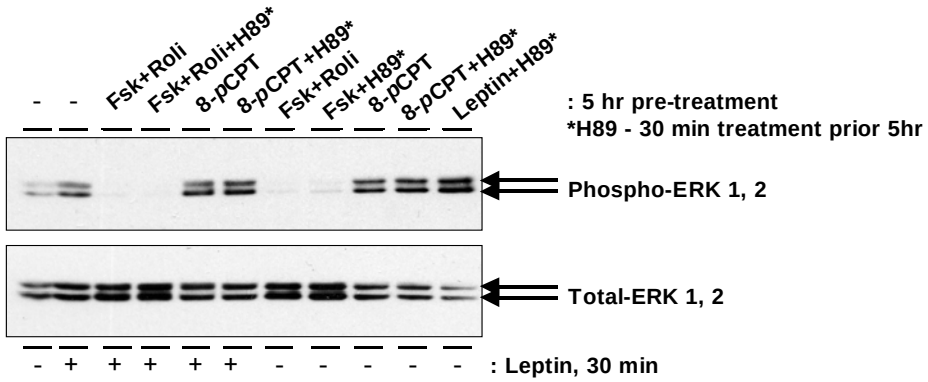

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
350
300
250
200
150
100
50
0
**
**
V
IL-6
IL-6 + F/R
IL-6 + F/R + H89
IL-6 + 8MC
IL-6 + 8MC + H89
F/R
F/R + H89
8MC
8MC + H89
IL-6 + H89
Treatment

Figure 4.15 The Effect of H89 on cAMP-mediated Inhibition of ERK1,2 Phosphorylation and the Effect of 8pCPT on ERK Phosphorylation by Leptin in AS-Ms

4 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 5μM H89 prior to treatment with 10μM forskolin (Fsk) and 10μM rolipram (Roli) or 100μM 8pCPT for 5 hrs in serum-free medium. Following 5 hrs, cells were treated with or without leptin at a concentration of 125ng/ml for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and serum-free medium at the same volume and for the same length of time as Fsk, Roli and leptin treatment respectively, and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 / **p<0.01 *versus* phosphorylated ERK1,2 levels in leptin-treated alone cells). Leptin-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli, 8MC=8pCPT].


- -
Fsk+Roli
Fsk+Roli+H89*
8-pCPT
8-pCPT+H89*
Fsk+Roli
Fsk+H89*
8-pCPT
8-pCPT+H89*
Leptin+H89*
: 5 hr pre-treatment
*H89 - 30 min treatment prior 5hr
Phospho-ERK 1, 2
Total-ERK 1, 2
- + + + + + - - - - -
: Leptin, 30 min

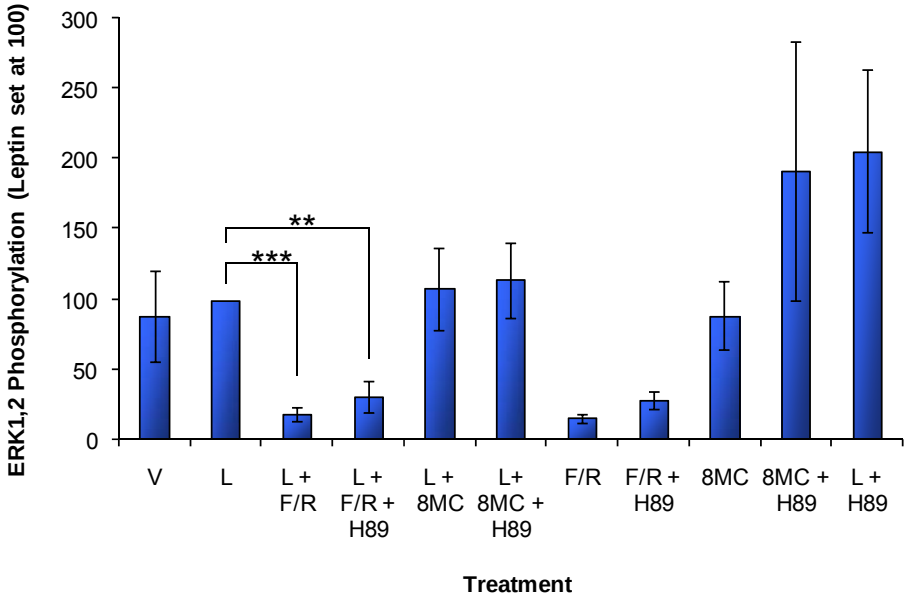
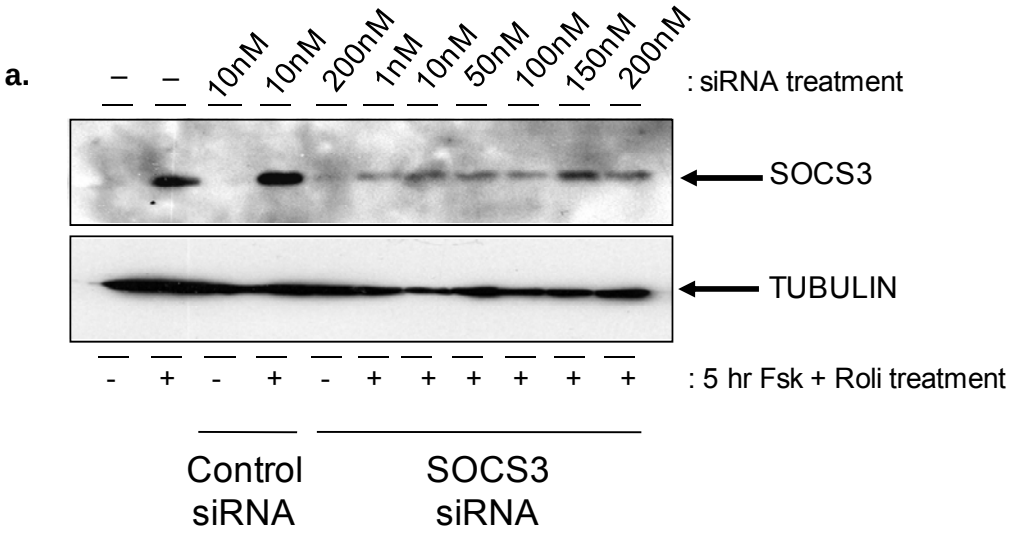

ERK1,2 Phosphorylation (Leptin set at 100)
300
250
200
150
100
50
0
**
***
V
L
L + F/R
L + F/R + H89
L + 8MC
L+ 8MC + H89
F/R
F/R + H89
8MC
8MC + H89
L + H89
Treatment

Figure 4.16 The Effect of Increasing Concentrations of SOCS3 siRNA on cAMP-mediated Induction of SOCS3 in HUVECs and AS-Ms

2×10^5 AS-Ms / well and 2×10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with or without 10nM control siRNA and varying concentrations of SOCS3 siRNA, and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. All wells were also treated with MG132 at a concentration of 6μM for 5 hrs. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. The immunoblots show the expression of SOCS3 in HUVECs (a.) and AS-Ms (b.) from one experiment. The expression of the cytoskeleton protein, tubulin is also shown in both HUVECs and ASM-S to control for protein loading.


a.
– – 10nM 10nM 200nM 1nM 10nM 50nM 100nM 150nM 200nM
: siRNA treatment
SOCS3
TUBULIN
- + - + - + + + + + +
: 5 hr Fsk + Roli treatment
Control siRNA
SOCS3 siRNA

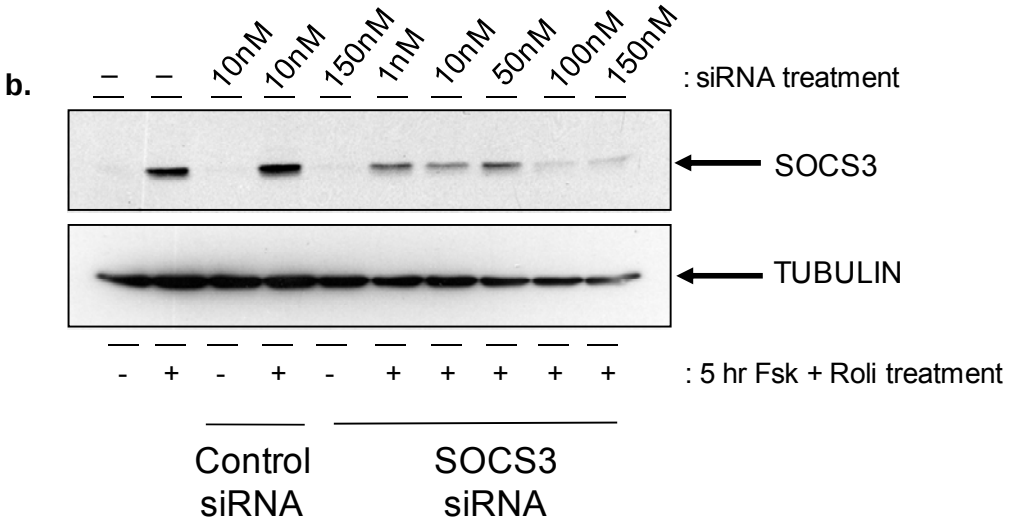
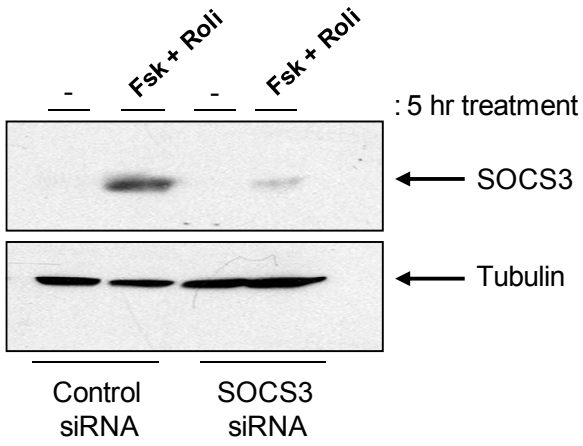

b.
– – 10nM 10nM 150nM 1nM 10nM 50nM 100nM 150nM
: siRNA treatment
SOCS3
TUBULIN
- + - + - + + + + +
: 5 hr Fsk + Roli treatment
Control siRNA
SOCS3 siRNA

Figure 4.17 The Effect of SOCS3 siRNA on cAMP-mediated Induction of SOCS3 in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Vehicle (V) treated cells were treated with ethanol and DMSO at the same volume and the same length of time as Fsk and Roli treatment respectively. All wells were also treated with MG132 at a concentration of 6μM for 5 hrs. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 expression levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* SOCS3 expression levels in vehicle-treated cells, *p<0.05 *versus* SOCS3 expression levels in Fsk/Roli-treated cells). Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


-
Fsk + Roli
-
Fsk + Roli
: 5 hr treatment
SOCS3
Tubulin
Control siRNA
SOCS3 siRNA

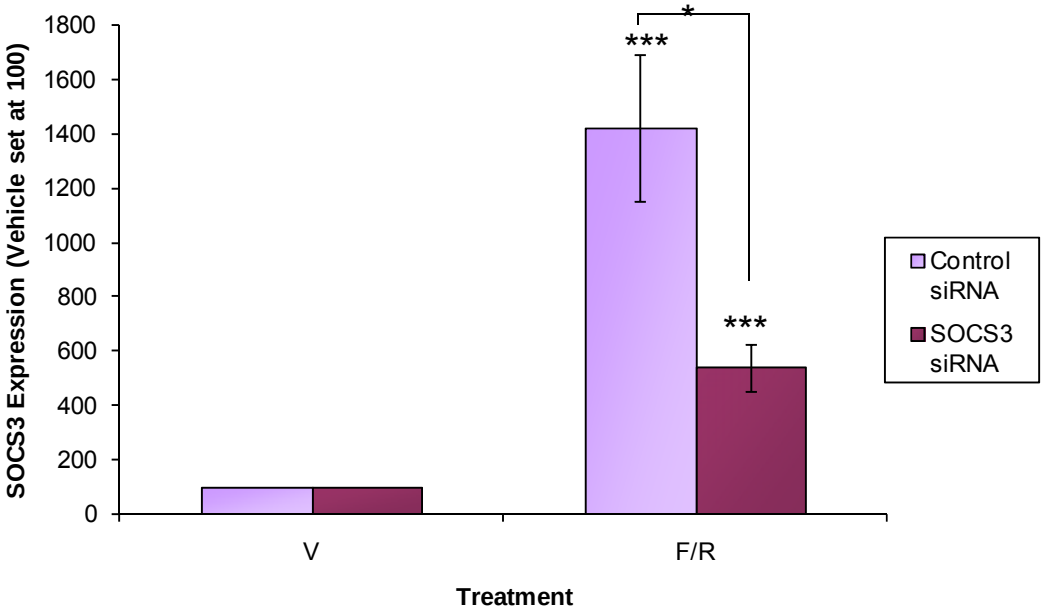
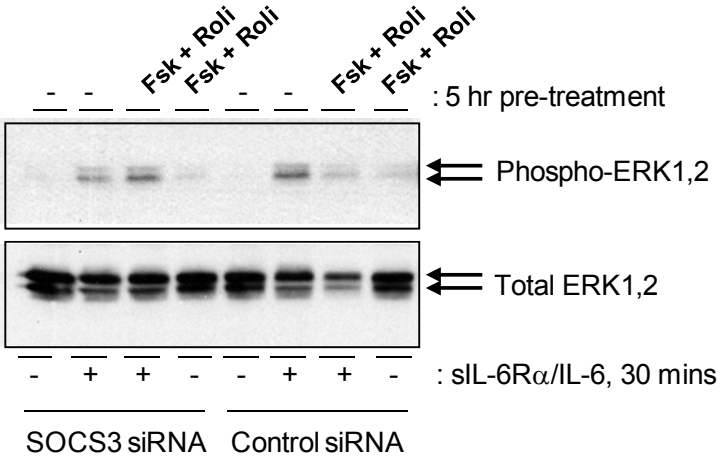

SOCS3 Expression (Vehicle set at 100)
1800
1600
1400
1200
1000
800
600
400
200
0
***
*
***
V
F/R
Treatment
Control siRNA
SOCS3 siRNA

Figure 4.18 The Effect of SOCS3 siRNA on cAMP-mediated Inhibition of ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 / **p<0.01 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells, ***p<0.001 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk + Roli Fsk + Roli - - Fsk + Roli Fsk + Roli
: 5 hr pre-treatment
Phospho-ERK1,2
Total ERK1,2
- + + - - + + -
: sIL-6Rα/IL-6, 30 mins
SOCS3 siRNA
Control siRNA

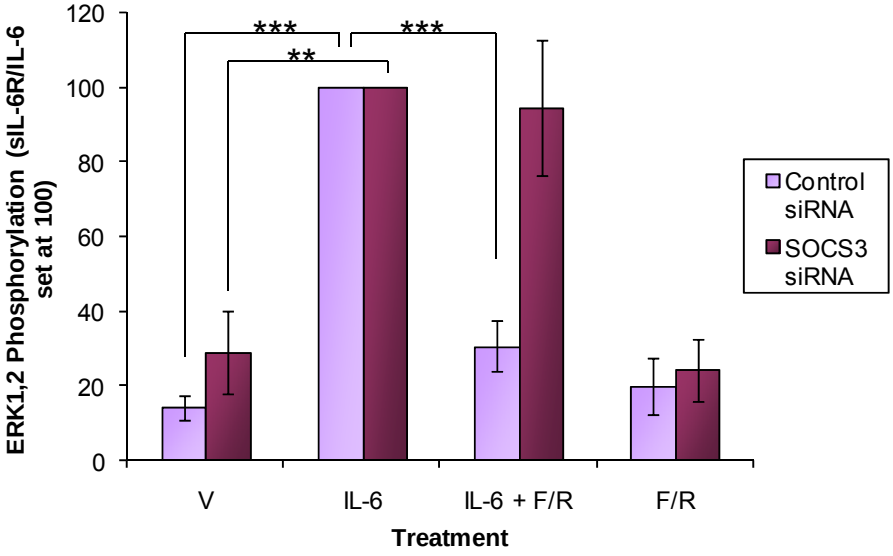
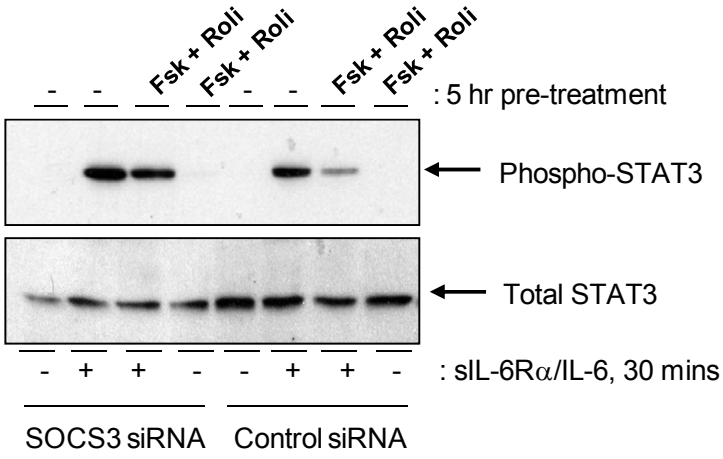

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
**
***
V
IL-6
IL-6 + F/R
F/R
Treatment
Control siRNA
SOCS3 siRNA

Figure 4.19 The Effect of SOCS3 siRNA on cAMP-mediated Inhibition of STAT3 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the third day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, ***p<0.001 / **p<0.01 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk + Roli Fsk + Roli - - Fsk + Roli Fsk + Roli
: 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + - - + + -
: sIL-6Rα/IL-6, 30 mins
SOCS3 siRNA
Control siRNA

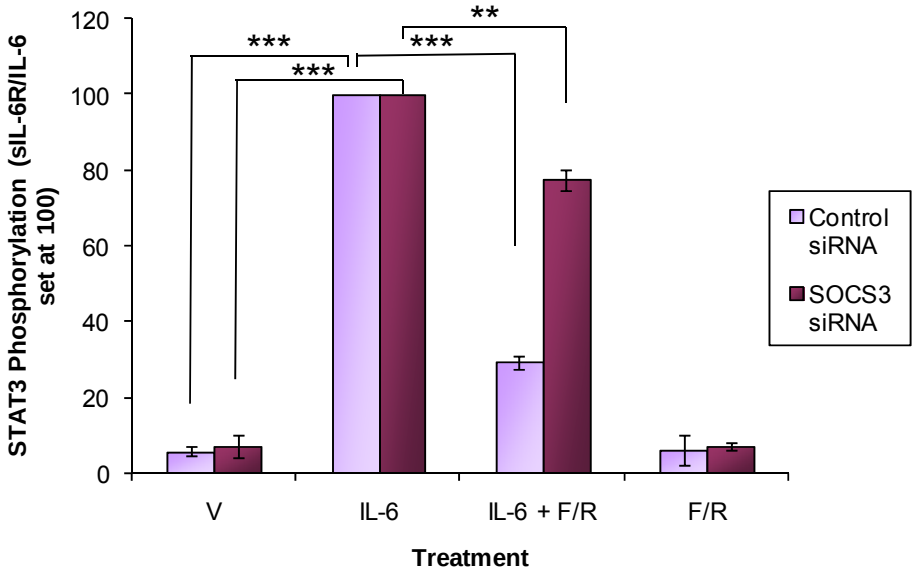
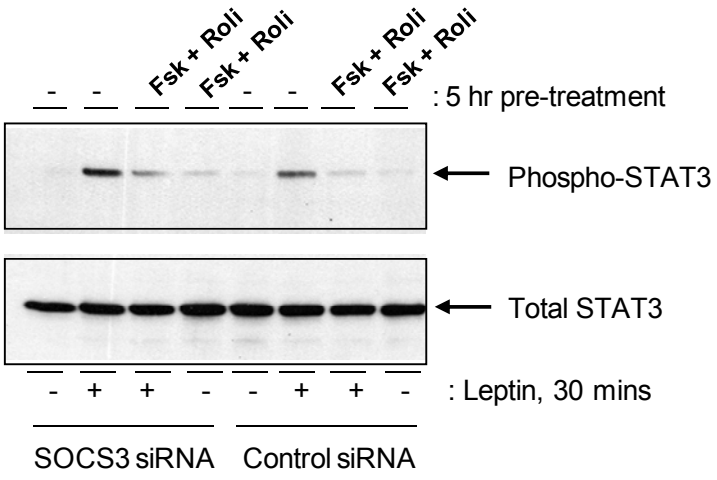

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
**
***
V
IL-6
IL-6 + F/R
F/R
Treatment
Control siRNA
SOCS3 siRNA

Figure 4.20 The Effect of SOCS3 siRNA on cAMP-mediated Inhibition of STAT3 Phosphorylation by Leptin in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the third day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without leptin at a concentration of 125ng/ml for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and serum-free medium at the same volume and for the same length of time as Fsk, Roli and leptin treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from four experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, ***p<0.001 *versus* phosphorylated STAT3 levels in leptin-treated alone cells). Leptin-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - - - - - - - : 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + - - + + - : Leptin, 30 mins
SOCS3 siRNA
Control siRNA

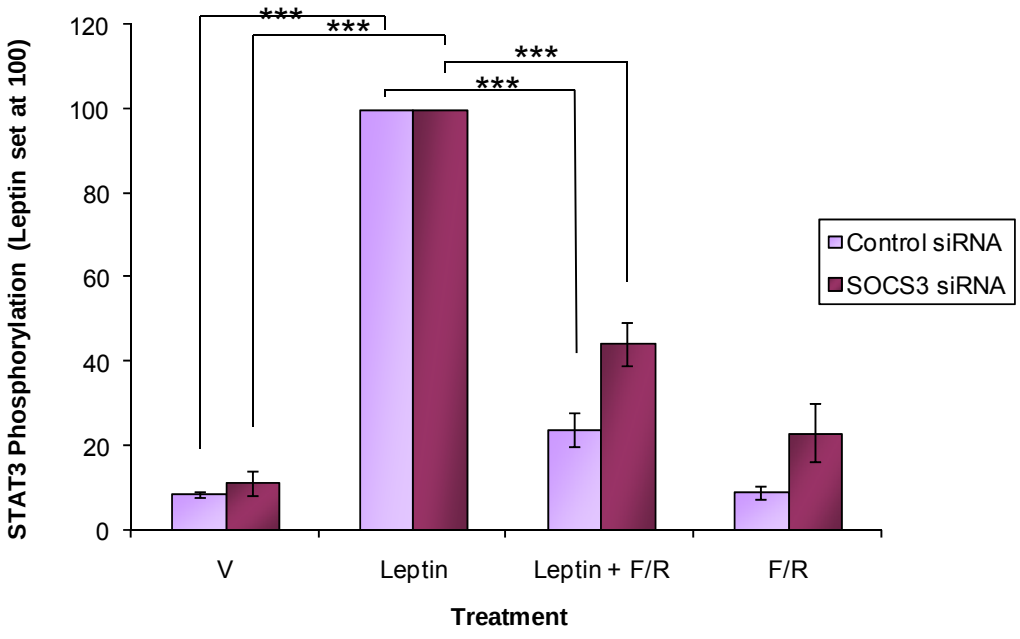

STAT3 Phosphorylation (Leptin set at 100)
120
100
80
60
40
20
0
***
***
***
***
V
Leptin
Leptin + F/R
F/R
Treatment
Control siRNA
SOCS3 siRNA

Figure 4.21 The Effect of SOCS3 siRNA on cAMP-mediated Induction of SOCS3 in AS-Ms

2 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Vehicle (V) treated cells were treated with ethanol and DMSO at the same volume and the same length of time as Fsk and Roli treatment respectively. All wells were also treated with MG132 at a concentration of 6μM for 5 hrs. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. The immunoblot shows the expression of SOCS3 in AS-Ms from one experiment. The expression of the cytoskeleton protein, tubulin is also shown as an immunoblot from 1 experiment to control for protein loading. [F=Fsk, R=Roli].

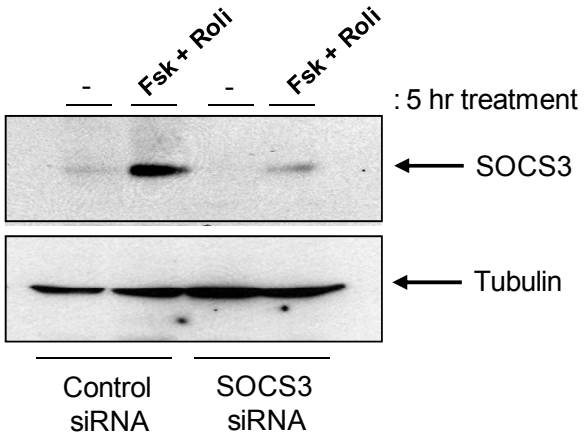
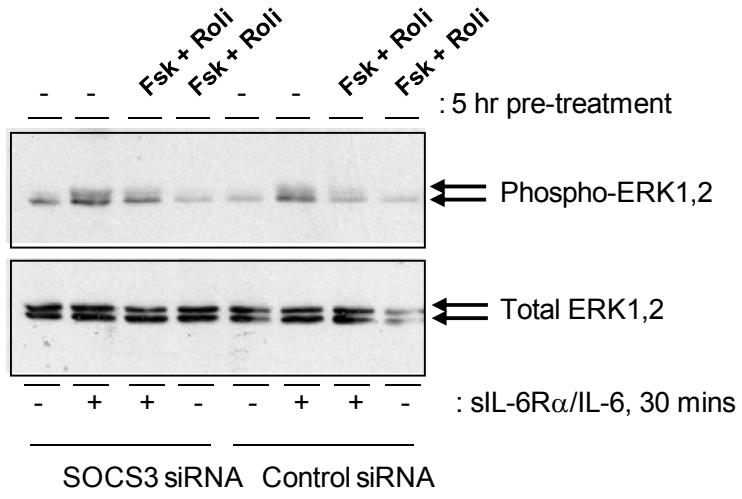

-
Fsk + Roli
-
Fsk + Roli
: 5 hr treatment
SOCS3
Tubulin
Control siRNA
SOCS3 siRNA

Figure 4.22 The Effect of SOCS3 siRNA on cAMP-mediated Inhibition of ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in AS-Ms

2 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the third day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively and 1μM PMA treated cells (data not shown), a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (**p<0.01 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells, ***p<0.001 / *p<0.05 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6 -treated alone cells). sIL-6Rα/IL-6 -treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - - - - - - -
: 5 hr pre-treatment
Phospho-ERK1,2
Total ERK1,2
- + + - - + + -
: sIL-6Rα/IL-6, 30 mins
SOCS3 siRNA
Control siRNA

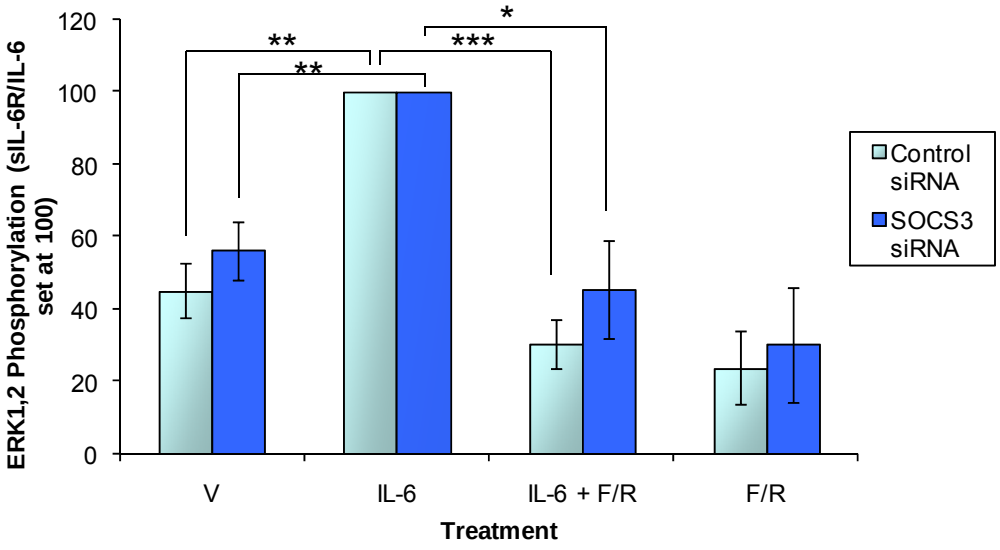
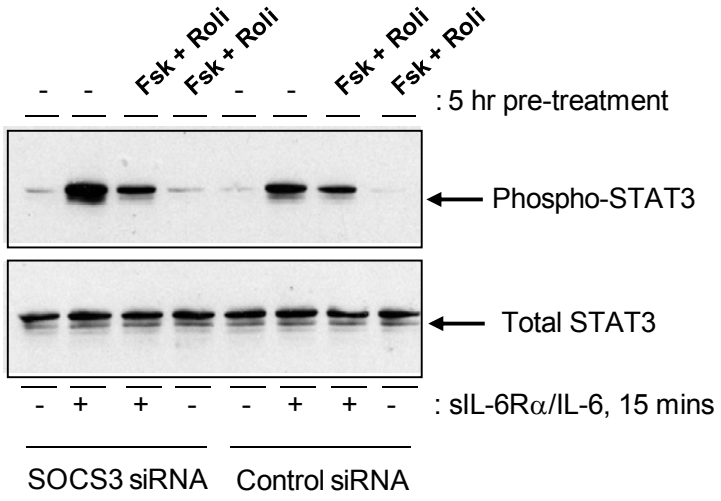

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
**
**
***
*
V
IL-6
IL-6 + F/R
F/R
Treatment
Control siRNA
SOCS3 siRNA

Figure 4.23 The Effect of SOCS3 siRNA on cAMP-mediated Inhibition of STAT3 Phosphorylation by sIL-6Rα/IL-6 in AS-Ms

2 x 10^5 AS-Ms / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 10nM control siRNA or 10nM SOCS3 siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the third day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in AS-Ms from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, *p<0.05 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6 -treated alone cells). sIL-6Rα/IL-6 -treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - - - - - - - : 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + - - + + - : sIL-6Rα/IL-6, 15 mins
SOCS3 siRNA
Control siRNA

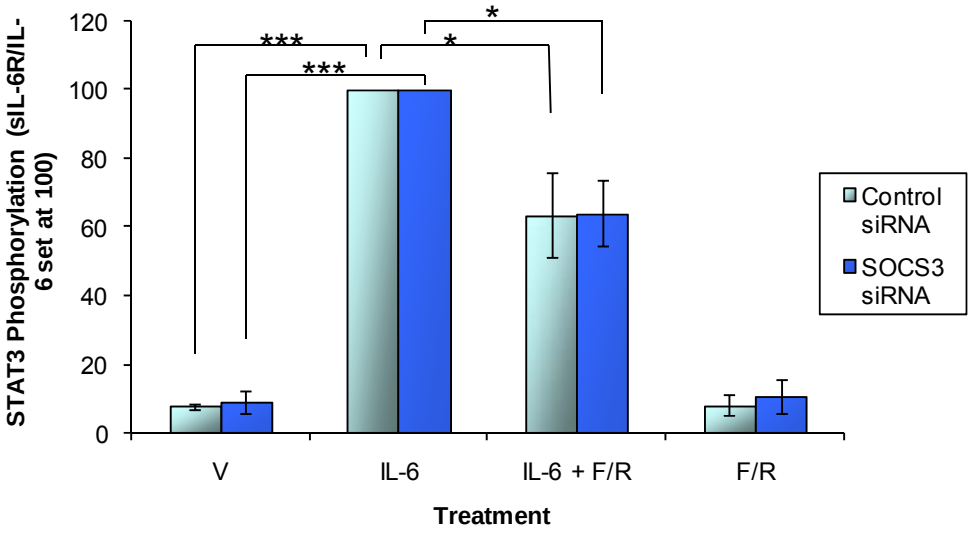
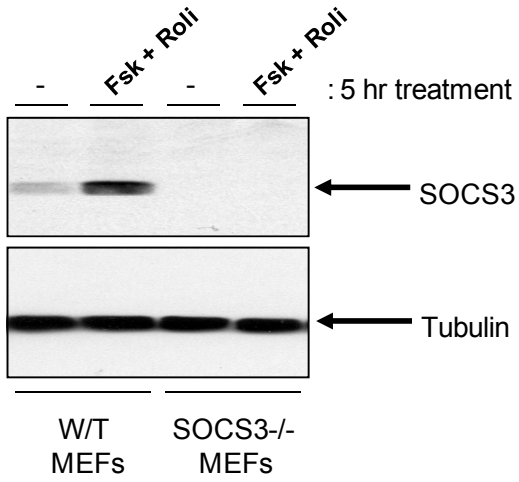

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
*
*
V
IL-6
IL-6 + F/R
F/R
Treatment
Control siRNA
SOCS3 siRNA

Figure 4.24 The Effect of cAMP elevating agents on SOCS3 Induction in W/T and SOCS3-/- MEFs

W/T and SOCS3-/- MEFs were seeded into 6 well plates and grown until confluent. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Vehicle (V) treated cells were treated with ethanol and DMSO at the same volume and for the same length of time as Fsk and Roli treatment respectively. All wells were also treated with MG132 at a concentration of 6μM for 5 hrs. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of SOCS3 expression levels in W/T and SOCS3-/- MEFs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* SOCS3 expression levels in vehicle-treated cells). Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


-
Fsk + Roli
-
Fsk + Roli
: 5 hr treatment
SOCS3
Tubulin
W/T MEFs
SOCS3-/- MEFs

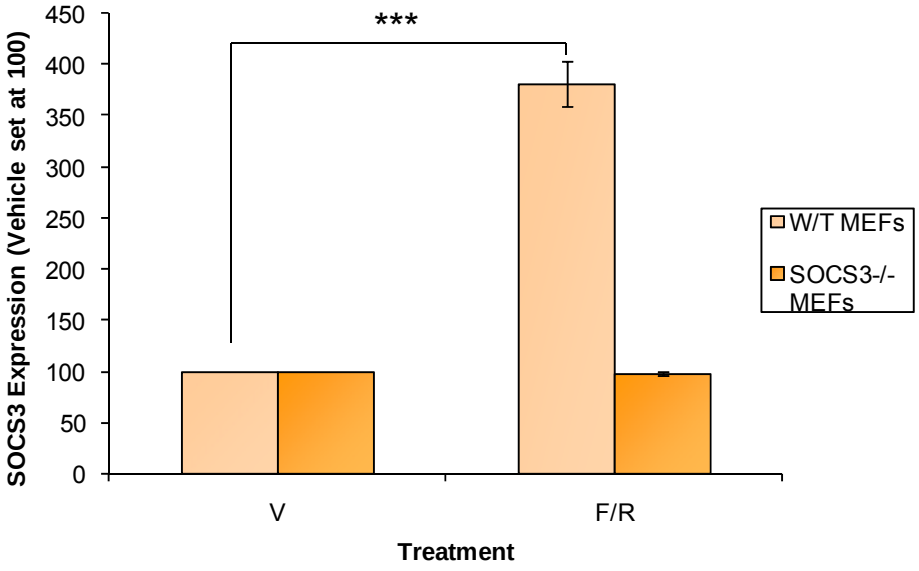
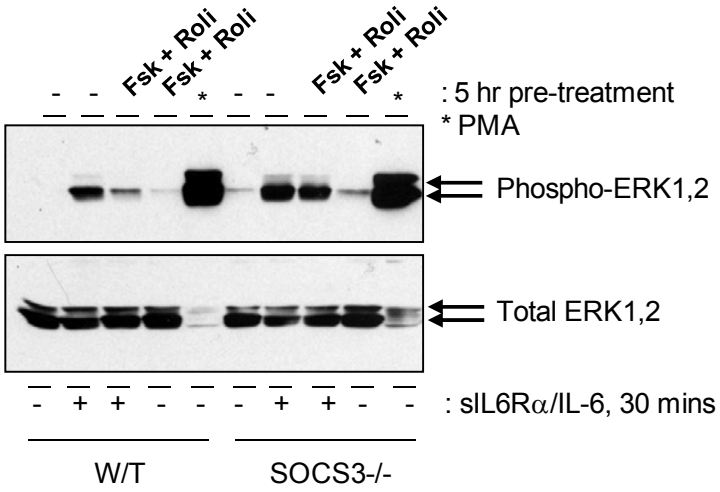

***
SOCS3 Expression (Vehicle set at 100)
450
400
350
300
250
200
150
100
50
0
V
F/R
Treatment
W/T MEFs
SOCS3-/- MEFs

Figure 4.25 The Effect of cAMP elevating agents on ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in W/T and SOCS3-/- MEFs

W/T and SOCS3-/- MEFs were seeded into 6 well plates and grown until confluent. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively, and 1μM PMA treated cells, a potent activator of ERK1,2 in endothelial cells (as observed in previous experiments in the Palmer lab). Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in W/T and SOCS3-/- MEFs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells, ***p<0.001 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - * - - * : 5 hr pre-treatment
* PMA
Phospho-ERK1,2
Total ERK1,2
- + + - - - + + - - : sIL6Rα/IL-6, 30 mins
W/T
SOCS3-/-

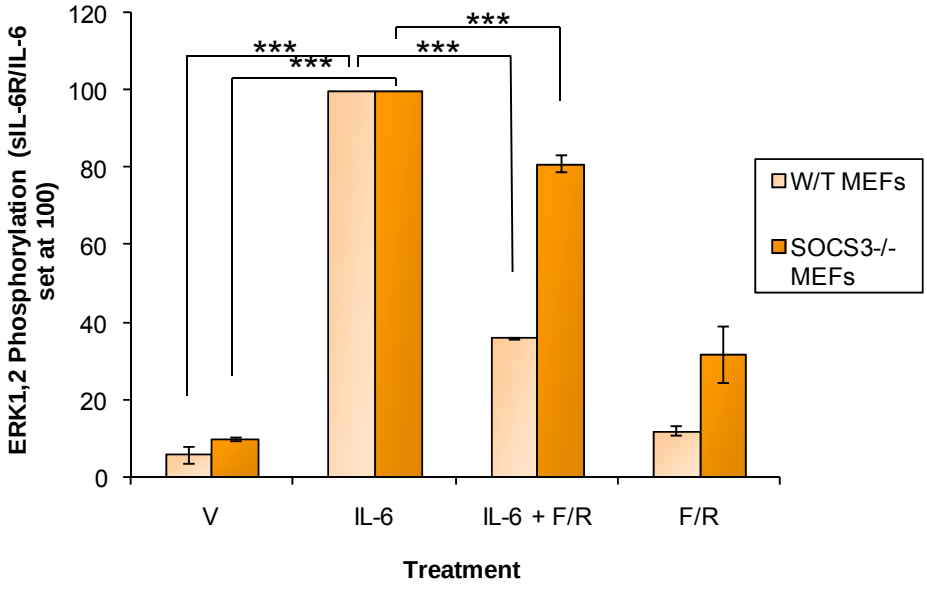
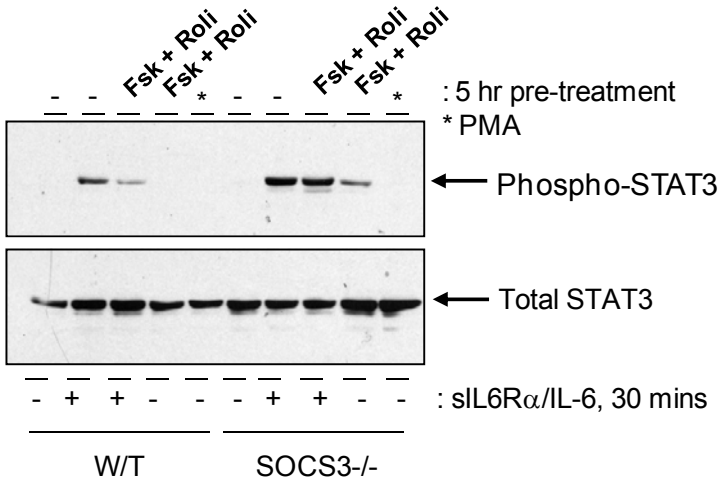

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
***
***
V
IL-6
IL-6 + F/R
F/R
Treatment
W/T MEFs
SOCS3-/- MEFs

Figure 4.26 The Effect of cAMP elevating agents on STAT3 Phosphorylation by sIL-6Rα/IL-6 in W/T and SOCS3-/- MEFs

W/T and SOCS3-/- MEFs were seeded into 6 well plates and grown until confluent. 24 hrs after seeding, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in W/T and SOCS3-/- MEFs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, *p<0.05 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6-treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - * - - * : 5 hr pre-treatment
* PMA
Phospho-STAT3
Total STAT3
- + + - - - + + - - : sIL6Rα/IL-6, 30 mins
W/T
SOCS3-/-

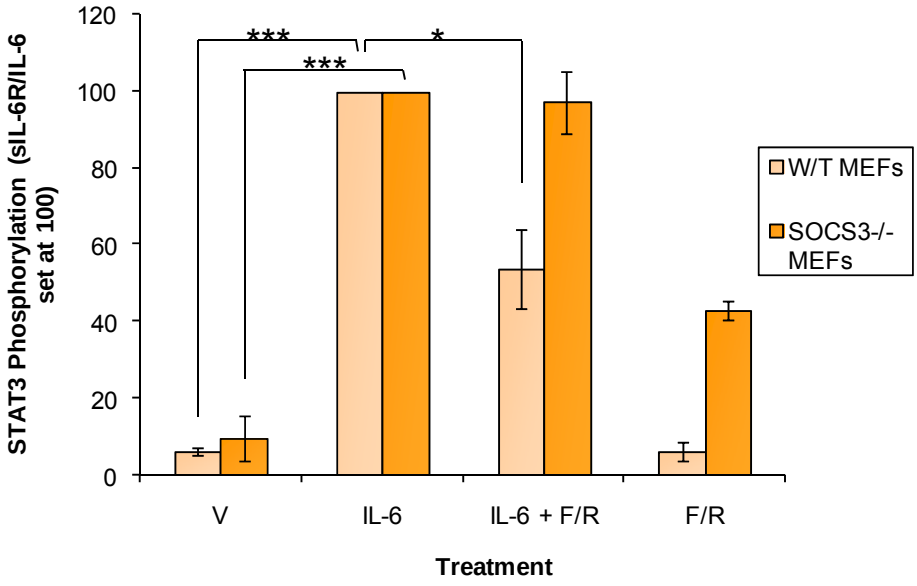
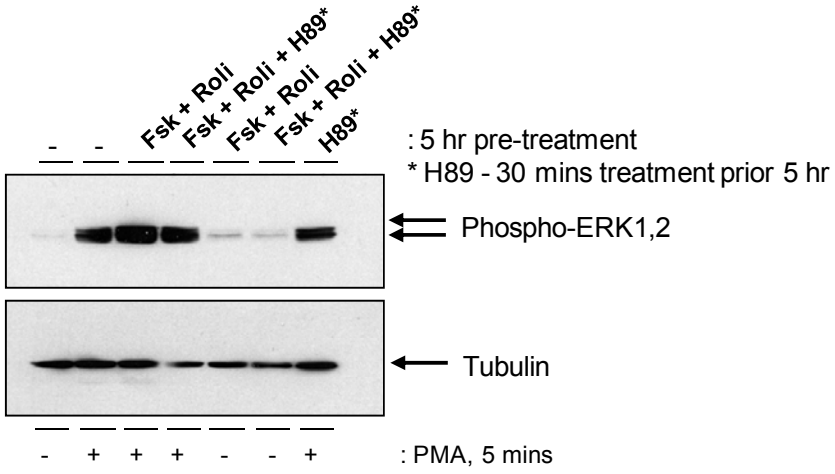

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
*
V
IL-6
IL-6 + F/R
F/R
Treatment
W/T MEFs
SOCS3-/- MEFs

Figure 4.27 The Effect of cAMP elevating agents (5 hr pre-treatment) in the presence and absence of H89 on ERK1,2 Phosphorylation by PMA in HUVECs

4 x 10^5 HUVECs were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 5μM H89 prior to treatment for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without 1 μM PMA for 5 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol and DMSO at the same volume and for the same length of time as Fsk, Roli and PMA treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 / *p<0.05 *versus* phosphorylated ERK levels in vehicle-treated cells). Basal is set at 100. The expression of the cytoskeleton protein, tubulin is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk + Roli Fsk + Roli + H89* Fsk + Roli Fsk + Roli + H89* H89*
: 5 hr pre-treatment
* H89 - 30 mins treatment prior 5 hr
Phospho-ERK1,2
Tubulin
- + + + - - +
: PMA, 5 mins

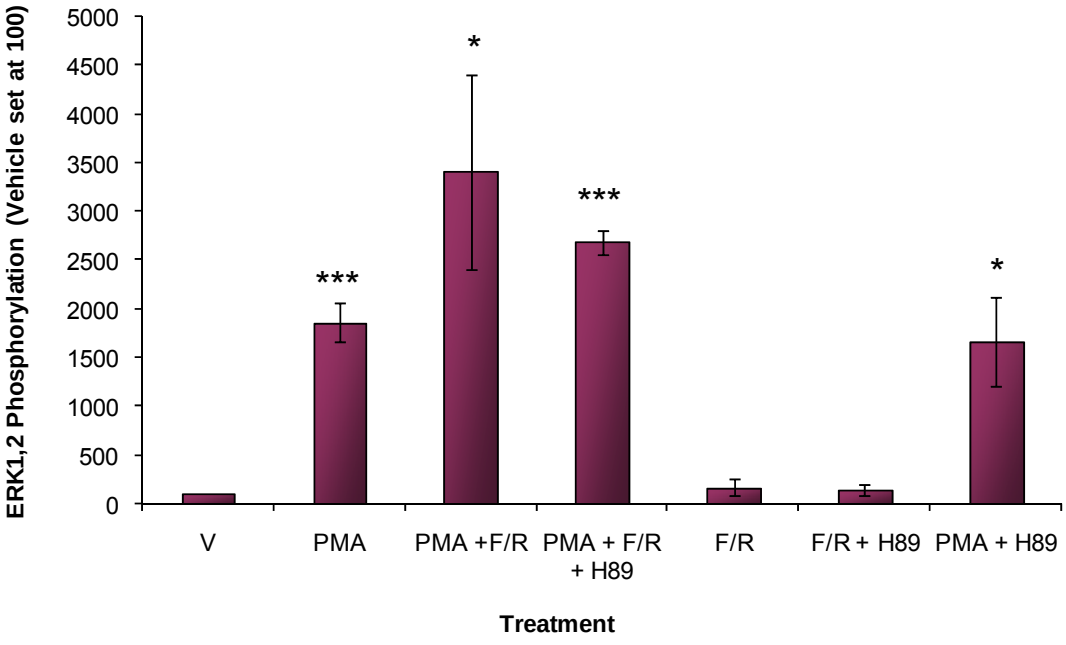
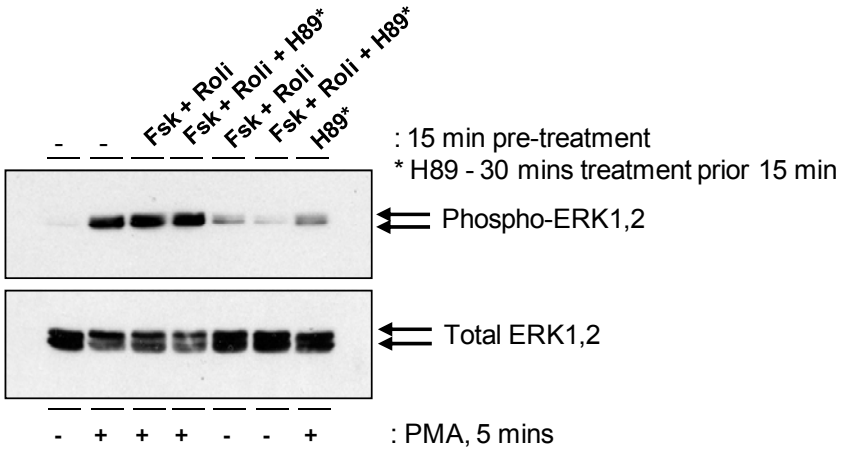

ERK1,2 Phosphorylation (Vehicle set at 100)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
***
***
*
V
PMA
PMA +F/R
PMA + F/R + H89
F/R
F/R + H89
PMA + H89
Treatment

Figure 4.28 The Effect of cAMP elevating agents (15 mins pre-treatment) in the presence and absence of H89 on ERK1,2 Phosphorylation by PMA in HUVECs

4 x 10^5 HUVECs were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 5μM H89 prior to treatment for 15 minutes with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 15 minutes, cells were treated with or without 1 μM PMA for 5 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol and DMSO at the same volume and for the same length of time as Fsk, Roli and PMA treatment. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 / **p<0.01 / *p<0.05 *versus* phosphorylated ERK levels in vehicle-treated cells, **p<0.01 *versus* phosphorylated ERK levels in PMA-treated alone cells, *p<0.05 *versus* phosphorylated ERK levels in Fsk- and Roli-treated alone cells). Basal is set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli + H89*
Fsk + Roli
Fsk + Roli + H89*
H89*
: 15 min pre-treatment
* H89 - 30 mins treatment prior 15 min
Phospho-ERK1,2
Total ERK1,2
- - + + + - - +
: PMA, 5 mins

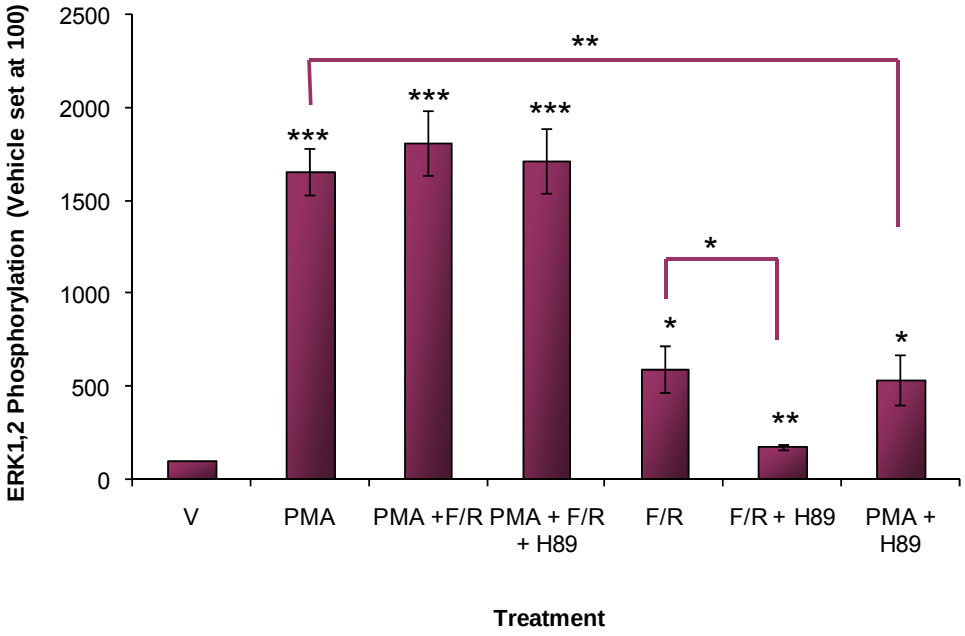
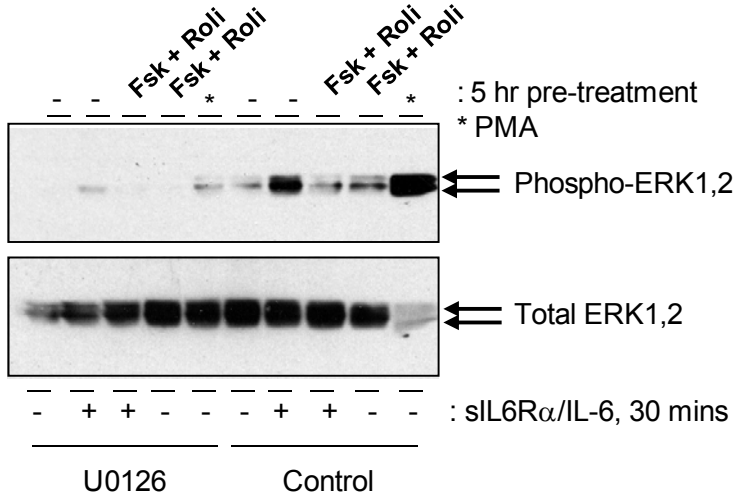

ERK1,2 Phosphorylation (Vehicle set at 100)
2500
2000
1500
1000
500
0
**
***
***
***
*
*
**
*
V
PMA
PMA +F/R
PMA + F/R + H89
F/R
F/R + H89
PMA + H89
Treatment

Figure 4.29 The Effect of U0126 Treatment on cAMP-mediated Inhibition of ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

4 x 10^5 HUVECs were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 1μM U0126 prior to treatment for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively, and 1μM PMA treated cells, a potent activator of ERK1,2 in endothelial cells. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 / *p<0.05 *versus* phosphorylated ERK levels in vehicle-treated cells, *p<0.05 *versus* phosphorylated ERK levels in sIL-6Rα/IL-6-treated alone cells). Basal is set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - * - - *
: 5 hr pre-treatment
* PMA
Phospho-ERK1,2
Total ERK1,2
- + + - - - + + - -
: sIL6Rα/IL-6, 30 mins
U0126
Control

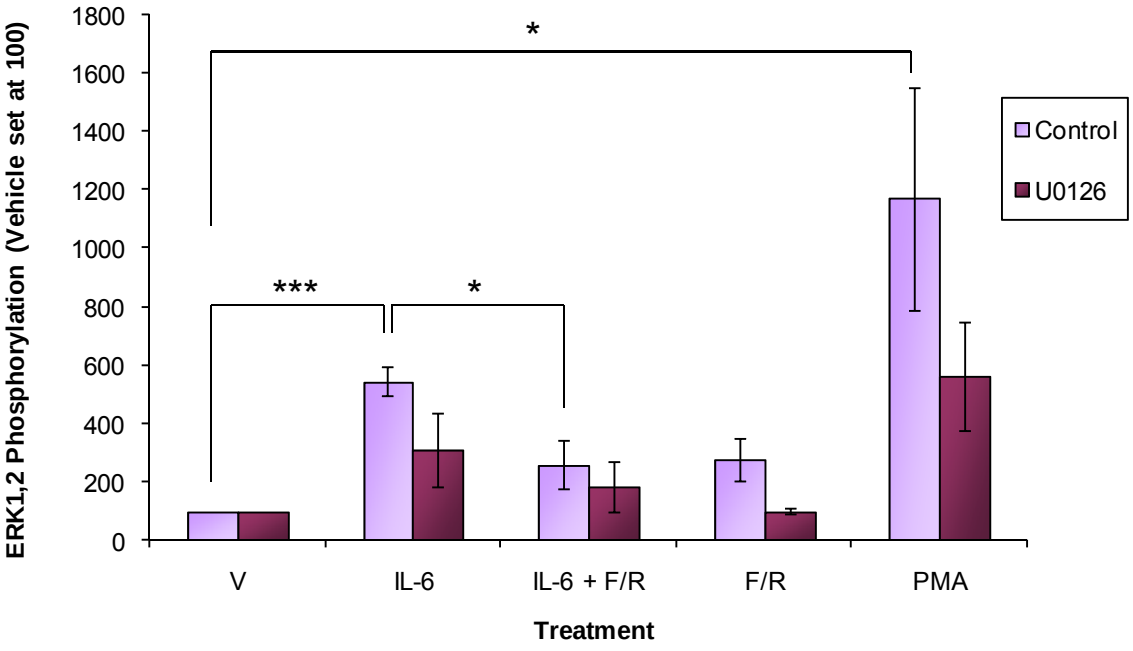
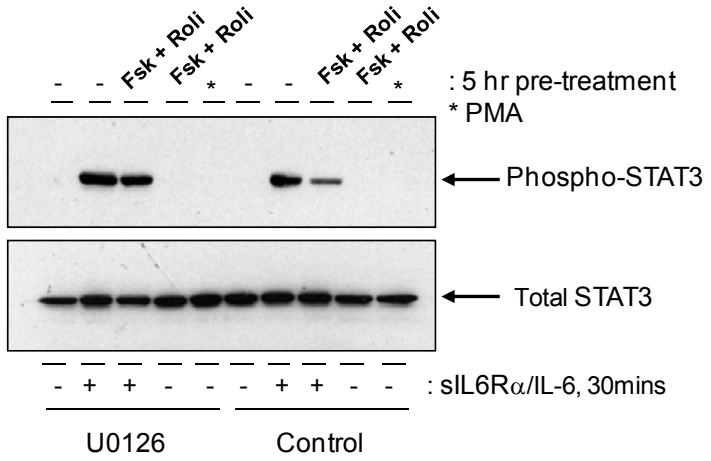

ERK1,2 Phosphorylation (Vehicle set at 100)
1800
1600
1400
1200
1000
800
600
400
200
0
*
***
*
Control
U0126
V
IL-6
IL-6 + F/R
F/R
PMA
Treatment

Figure 4.30 The Effect of U0126 Treatment on cAMP-mediated Inhibition of STAT3 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

4 x 10^5 HUVECs were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 1μM U0126 prior to treatment for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Following 5 hrs, cells were treated with or without sIL-6Rα/IL-6 at a concentration of 25ng/ml/5ng/ml respectively for 30 minutes. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol, DMSO and PBS at the same volume and for the same length of time as Fsk, Roli and sIL-6Rα/IL-6 treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from three experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells, ***p<0.001 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6-treated alone cells). Basal is set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


- - Fsk + Roli Fsk + Roli * - - Fsk + Roli Fsk + Roli *
: 5 hr pre-treatment
* PMA
Phospho-STAT3
Total STAT3
- + + - - - + + - -
: sIL6Rα/IL-6, 30mins
U0126
Control

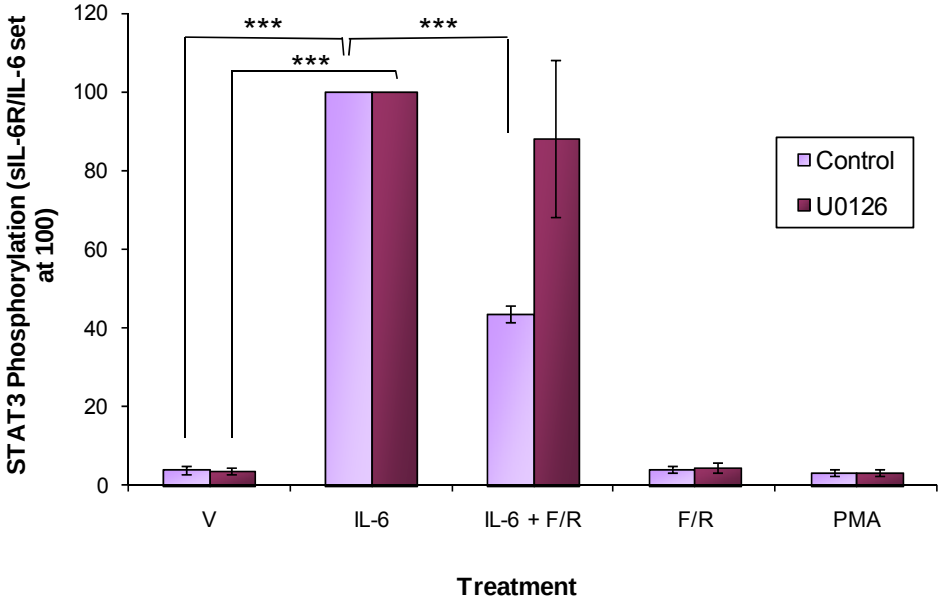
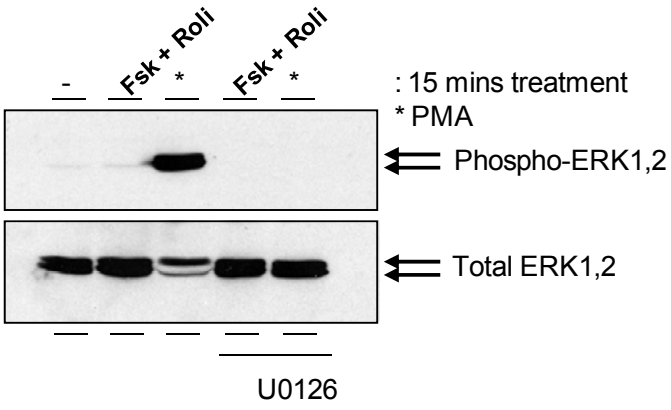

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
***
Control
U0126
V
IL-6
IL-6 + F/R
F/R
PMA
Treatment

Figure 4.31 The Effect of cAMP elevating agents on ERK1,2 Phosphorylation in AS-Ms

4 x 10^5 AS-Ms were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with PBS and treated for 30 minutes with or without 1μM U0126 prior to treatment for 15 mins with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli), in serum-free medium. The controls in this experiment included vehicle (V) treated cells, which were treated with ethanol and DMSO at the same volume and for the same length of time as Fsk and Roli treatment respectively. Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in AS-Ms from three experiments is presented as mean values +/- standard error (*p<0.05 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells). Basal is set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading. [F=Fsk, R=Roli].


-
Fsk + Roli
*
Fsk + Roli
*
: 15 mins treatment
* PMA
Phospho-ERK1,2
Total ERK1,2
U0126

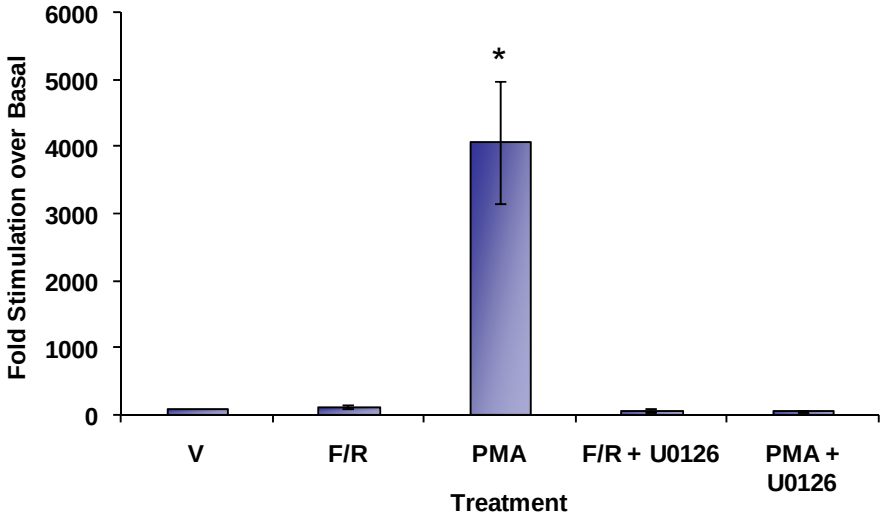
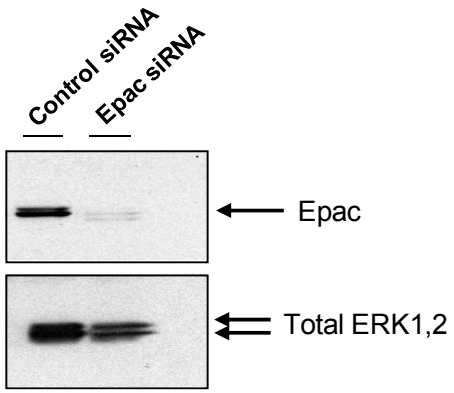

6000
5000
4000
3000
2000
1000
0
Fold Stimulation over Basal
*
V
F/R
PMA
F/R + U0126
PMA + U0126
Treatment

Figure 4.32 The Effect of Epac siRNA on basal levels of Epac Expression in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 20nM control siRNA or 20nM Epac siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and incubated for 5 hrs in serum-free medium. Following incubation, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of Epac expression levels in HUVECs from four experiments is presented as mean values +/- standard error (***p<0.001 *versus* Epac expression levels in control siRNA-treated cells). Control siRNA-treated cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading.


Control siRNA
Epac siRNA
Epac
Total ERK1,2

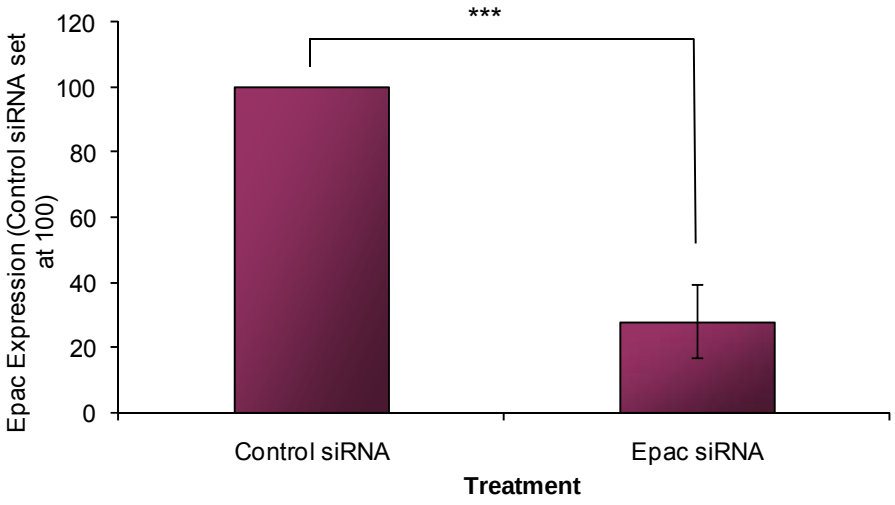
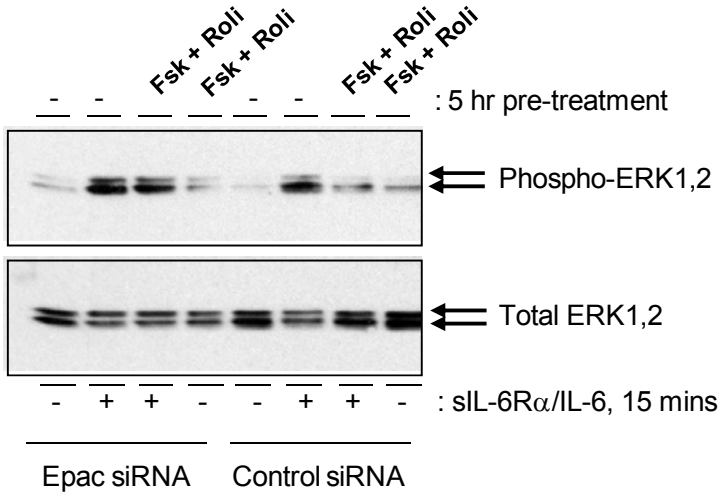

***
120
100
80
60
40
20
0
Epac Expression (Control siRNA set at 100)
Control siRNA
Epac siRNA
Treatment

Figure 4.33 The Effect of Epac siRNA on cAMP-mediated Inhibition of ERK1,2 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 20nM control siRNA or 20nM Epac siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Vehicle (V) treated cells were treated with ethanol and DMSO at the same volume and the same length of time as Fsk and Roli treatment respectively Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated ERK1,2 levels in HUVECs from five experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated ERK1,2 levels in sIL-6Rα/IL-6 -treated alone cells, **p<0.01 / *p<0.05 *versus* phosphorylated ERK1,2 levels in vehicle-treated cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total ERK1,2 expression is also shown as a representative immunoblot to control for protein loading.


Fsk + Roli
Fsk + Roli
Fsk + Roli
Fsk + Roli
- - - - - - - - : 5 hr pre-treatment
Phospho-ERK1,2
Total ERK1,2
- + + - - + + - : sIL-6Rα/IL-6, 15 mins
Epac siRNA
Control siRNA

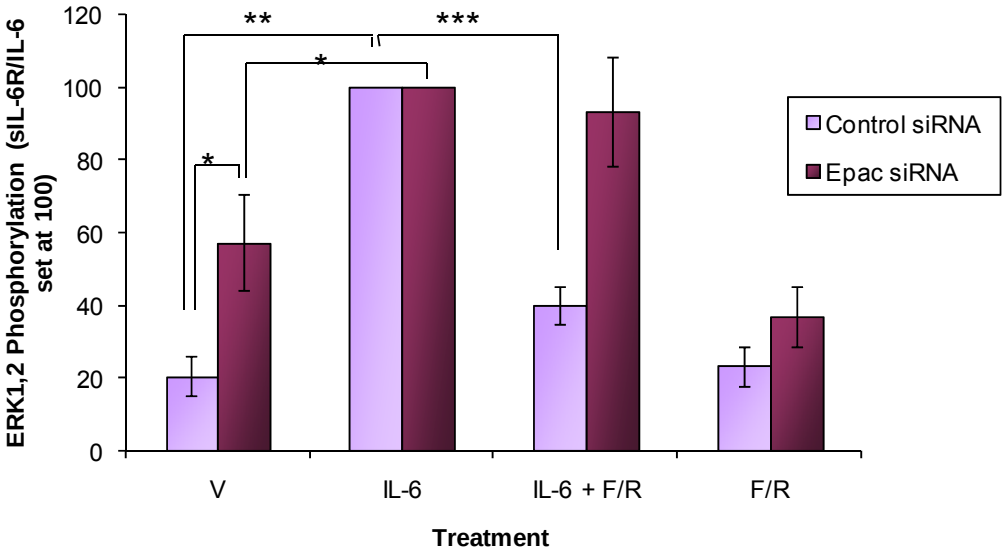
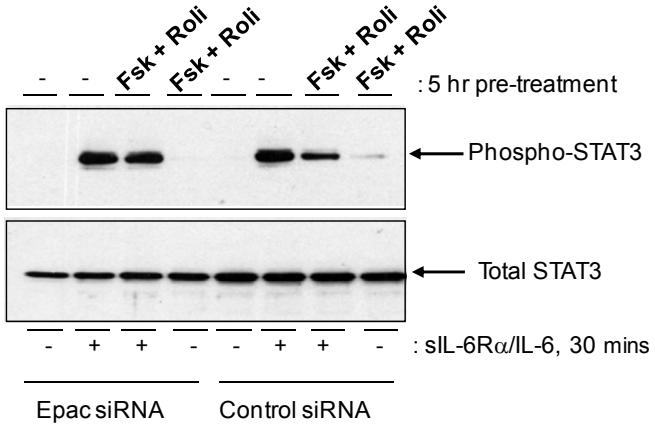

ERK1,2 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
**
***
*
*
Control siRNA
Epac siRNA
V
IL-6
IL-6 + F/R
F/R
Treatment

Figure 4.34 The Effect of Epac siRNA on cAMP-mediated Inhibition of STAT3 Phosphorylation by sIL-6Rα/IL-6 in HUVECs

2 x 10^5 HUVECs / well were seeded into 6 well plates. 24 hrs after seeding, cells were washed twice with serum-free medium before adding 1ml / well serum-free medium. Cells were then transfected with either 20nM control siRNA or 20nM Epac siRNA and incubated for 5 hrs. After 5 hrs, 1ml / well medium was added to the cells for incubation overnight. The following day, the transfection procedure was repeated. On the fourth day, cells were washed twice with PBS and treated for 5 hrs with or without 10μM forskolin (Fsk) and 10μM rolipram (Roli) in serum-free medium. Vehicle (V) treated cells were treated with ethanol and DMSO at the same volume and the same length of time as Fsk and Roli treatment respectively Following treatment, soluble cell extracts equalised for protein concentration were fractionated by SDS-PAGE for immunoblotting with the indicated antibodies. Quantitative analysis of phosphorylated STAT3 levels in HUVECs from four experiments is presented as mean values +/- standard error (***p<0.001 *versus* phosphorylated STAT3 levels in vehicle-treated cells and sIL-6Rα/IL-6 -treated alone cells, *p<0.05 *versus* phosphorylated STAT3 levels in sIL-6Rα/IL-6 -treated alone cells). sIL-6Rα/IL-6-treated alone cells set at 100. Total STAT3 expression is also shown as a representative immunoblot to control for protein loading.


- - Fsk + Roli Fsk + Roli - - Fsk + Roli Fsk + Roli
: 5 hr pre-treatment
Phospho-STAT3
Total STAT3
- + + - - + + - : sIL-6Rα/IL-6, 30 mins
Epac siRNA
Control siRNA

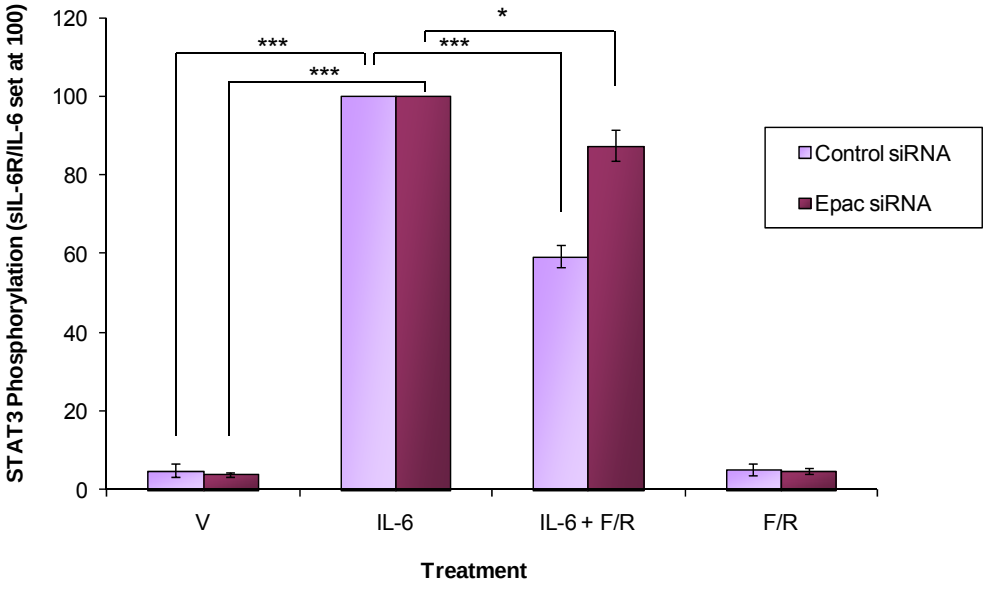
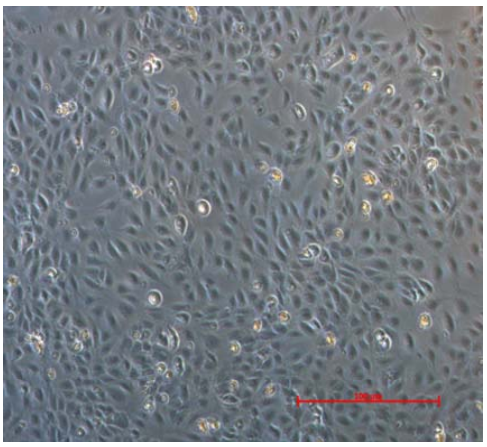

STAT3 Phosphorylation (sIL-6R/IL-6 set at 100)
120
100
80
60
40
20
0
***
***
***
*
V
IL-6
IL-6 + F/R
F/R
Treatment
Control siRNA
Epac siRNA

Figure 4.35 HUVEC Morphology in Response to SOCS3 and Epac siRNA Treatment

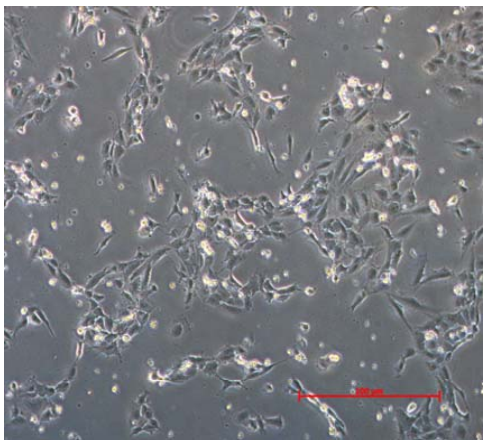
Light micrograph images of HUVECs treated with and without Epac siRNA (a) and SOCS3 siRNA (b) at day 4 of the transfection process. Images were captured using a Zeiss microscope (10 x objective lens) and manipulated using Axiovision software.

a.

Control siRNA

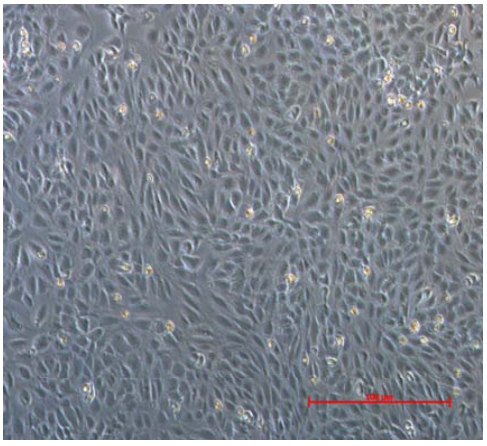


Epac siRNA

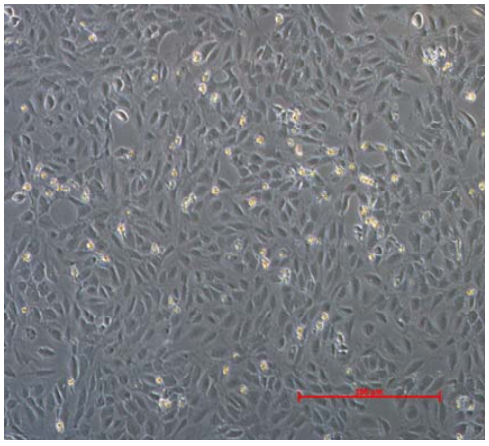


b.

Control siRNA



SOCS3 siRNA



Chapter 5

Final Discussion

Summary

An exhaustive number of studies have demonstrated the inhibitory effects of cAMP on a myriad of cell types and tissues. Significantly, elevation of cAMP has been associated with the inhibition of function of various inflammatory cell types, including macrophages, neutrophils, eosinophils, T cells and endothelial cells (Bulut *et al.*, 1993; Dent *et al.*, 1991; Fonteh *et al.*, 1993; Nielson, 1987; Pober *et al.*, 1993; Renz *et al.*, 1988; Teixeira *et al.*, 1997). Examples of these inhibitory effects include, the reduction of cytokines, such as TNFα, and nitric oxide release from macrophages (Bulut *et al.*, 1993; Renz *et al.*, 1988), the suppression of lysosomal enzymes, ROS and platelet-activating factor (PAF) from neutrophils (Fonteh *et al.*, 1993; Nielson, 1987), the inhibition of eosinophil respiratory burst activity (Dent *et al*., 1991), the inhibition of cytotoxic T lymphocyte activation (Teixeira *et al.*, 1997; Wisloff & Christoffersen, 1977), and importantly, the inhibition of TNFα-induced adhesion molecule expression in endothelial cells (Morandini *et al.*, 1996; Pober *et al.*, 1993). Furthermore, studies have also shown a cAMP-mediated reduction in endothelial cell permeability (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005; Suttorp *et al.*, 1993), and suppression of transendothelial leukocyte migration (Lidington *et al.*, 1996; Lorenowicz *et al.*, 2007).

PDE4 inhibitors have been extensively studied in various animal models of inflammatory disease (reviewed in Teixeira *et al*., 1997). All of these studies have demonstrated suppression of inflammation and disease activity following treatment with PDE4 inhibitors (Teixeira *et al*., 1997). Clinically, PDE4 inhibitors have been in development since the early 1980s, with asthma and chronic obstructive pulmonary disease (COPD) constituting the predominant disease indications. However, not one of these PDE4 inhibitors has yet reached the market. This is due to lack of efficacy and/or dose-limiting adverse effects, such as nausea, abdominal pain, vomiting and diarrhea (Giembycz, 2008). An example of one such PDE4 inhibitor is rolipram, which has been used throughout the course of the current study. This was terminated during clinical development due to its side effects of nausea and gastrointestinal disturbances (Boswell-Smith *et al*., 2006). It is believed that the inhibition of PDE4D in particular, in non-target tissues, is responsible for the emetic effects. Since PDE4 is encoded by four genes (A-D), selective sub-type inhibitors could potentially overcome the side effects. Indeed,

selective ablation of PDE4B in mouse macrophages has been shown to substantially reduce LPS-stimulated TNFα release, whereas PDE4A and PDE4D-null macrophages displayed no reduction in LPS-stimulated TNFα release. Furthermore, PDE4B null mice were partially protected from LPS-induced shock (Jin *et al*., 2005). In addition to sub-type specific inhibitors, slow–releasing formulations have been developed to reduce systemic exposure. For example, pentoxifylline is a slow-releasing non-selective PDE inhibitor, which has been used to treat peripheral vascular disease and cerebrovascular disease (Ward & Clissold, 1987).

With regards to the clinical implications of inhibiting cytokine signalling, a humanised anti-IL-6R monoclonal antibody so-called tocilizumab has been used in the treatment of rheumatoid arthritis (RA) with strong therapeutic effects (Nakahara & Nishimoto, 2006). Tocilizumab has also been used in Crohn's disease and Castleman's disease with promising results (Ito *et al.*, 2004; Nakahara & Nishimoto, 2006). Interestingly, a phase III study is currently recruiting participants to investigate the effect of tocilizumab on lipids, arterial stiffness and markers of atherogenic risk in patients with moderate to severe active rheumatoid arthritis (www.clinicaltrials.gov). Therapeutically, the inhibition of leptin signalling is more complex. Human leptin deficiency is associated with morbid obesity. Despite this obesity, no risk factors for cardiovascular disease, such as hypertension and impairment in lipid metabolism have been observed (Ozata *et al*., 1999). This supports the presence of selective resistance in obese (non-leptin deficient) individuals, wherein these individuals display central but not peripheral resistance to leptin, as discussed in Section 1.3.6, Chapter 1 Introduction. For example, *ob/ob* mice on an atherogenic diet are protected from atherosclerosis, despite displaying obesity, diabetes, and hyperlipidemia (Schafer *et al*., 2004). Indeed, leptin has been shown to promote atherosclerosis and thrombus formation in atherosclerotic-prone apoE-deficient mice, despite a reduction in adipose tissue mass and fasting insulin levels (Bodary *et al*., 2005). Therefore, hyperleptinemia may be the contributory factor that leads to the cardiovascular morbidity associated with obesity. Inhibiting leptin signalling may represent a promising strategy for limiting the progression of atherosclerosis in hyperleptinemic obese individuals.

Thus, the inhibition of IL-6 signalling has demonstrated clinically beneficial effects on inflammatory diseases, such as RA and Crohn's disease. Furthermore, human epidemiological studies have shown that leptin is associated with the development of cardiovascular disease (Ciccone *et al.*, 2001; Wannamethee *et al.*, 2007; Wolk *et al.*, 2004). In addition, research has shown that both cytokines can modulate EC function, for example, by upregulating adhesion molecules such as VCAM-1 and ICAM-1, increasing MCP-1 and endothelin-1 expression, and inducing oxidative stress (Modur *et al.*, 1997; Quehenberger *et al.*, 2002; Romano *et al.*, 1997; Yamagishi *et al.*, 2001). As such, it would be of interest to further investigate ways in which these signalling pathways can be negatively regulated. As mentioned previously, cAMP is considered a crucial immunomodulator. Although the anti-inflammatory effects of cAMP have been well described, the molecular mechanisms underlying these effects are less known. In relation to endothelial cells, cAMP has been shown to limit vascular permeability and enhance endothelial barrier function *via* a cAMP/Epac/Rap1 pathway (Cullere *et al.*, 2005; Fukuhara *et al.*, 2005). This is of particular relevance to the present study as results have suggested an Epac1-dependent cAMP-mediated inhibition of IL-6/sIL-6α-stimulated ERK1,2 and STAT3 activation in HUVECs. Furthermore, the present results demonstrate the involvement of SOCS3 in the observed inhibition. Therefore, an Epac1- and SOCS3-dependent inhibitory pathway has been identified and represents a novel inhibitory pathway, distinct from the PKA-Raf1 pathway which has been well described for the cAMP-mediated inhibition of growth factor signalling. This novel pathway is important in terms of expanding our knowledge of endogenous protective signalling pathways that can potentially be exploited for therapeutic benefit. Interestingly, intracellular delivery of SOCS3 has been shown to greatly attenuate the acute inflammation observed following administration of staphylococcal enterotoxin B (SEB) and lipopolysaccaride (LPS) in mice. A cell-penetrating form of SOCS3 (CP-SOCS3) has been developed, which has a membrane-translocating motif (MTM) derived from the hydrophobic signal sequence of fibroblast growth factor 4, attached to either the N-terminal or C-terminal end. CP-SOCS3 administered to mice challenged with SEB or LPS *via* intraperitoneal injection revealed that CP-SOCS3 increased survival and suppressed inflammatory cytokines, such as TNFα and IL-6 (Jo *et al*., 2005). In a

similar study, liposome-mediated gene delivery of SOCS3 in a lethal endotoxic shock mouse model was shown to increase survival and greatly reduce the serum level of TNFα from peritoneal macrophages (Fang *et al*., 2005).

In contrast, a role for SOCS3 in the cAMP-mediated inhibition of leptin-stimulated STAT3 activation in HUVECs could not be demonstrated. As an alternative to SOCS3; SHP2, PTPB1 and other SOCS members may be responsible for the observed inhibition (as discussed in Chapter 4 Discussion). These results are in contrast to studies using mice with a neuronal deletion of SOCS3, which demonstrated prolonged activation of STAT3 in response to leptin. Moreover, SOCS3 deficient mice exhibited greater body weight loss when compared to their wild-type littermates and were also resistant to high fat diet-induced weight gain and hyperleptinemia, and retained insulin sensitivity (Mori *et al*., 2004). Although SOCS3 has been implicated in the negative regulation of leptin signalling, a large body of research has also implicated PTPB1 in this inhibition, which is discussed in Chapter 4 Discussion. Interestingly, PTP1B has been shown to target the insulin receptor as well as JAK2, contributing towards insulin resistance in addition to leptin resistance (Lavens *et al*., 2006). Inhibitors of PTP1B are currently under investigation for the treatment of obesity and type 2 diabetes (Hooft van Huijsduijnen *et al*., 2002). Indeed, further research will need to be carried out to confirm the involvement of PTPB1 in cAMP-mediated inhibition of leptin signalling in HUVECs observed in the present study, for example, by using siRNA knock down or over expression approaches. Of note, SOCS3 has also been shown to target insulin signalling *via* ubiquitin-mediated degradation of IRS1 and IRS2 in HEK293 cells (Rui *et al*., 2002). As IRS1 and IRS2 represent alternative SOCS binding partners, it may be of interest to determine whether or not the Epac1/SOCS3 pathway observed in the present study also regulates the SOCS3-mediated degradation of IRS1 and IRS2.

Consistent with the SOCS3-independent cAMP-mediated inhibition of leptin-stimulated STAT3 activation in HUVECs, experiments conducted in AS-Ms demonstrated no involvement of SOCS3 in the cAMP-mediated inhibition of sIL-6Rα/IL-6-induced STAT3 and ERK1,2 activation. As previously mentioned, other inhibitory mechanisms independent of SOCS3 may be responsible for the observed inhibition, such as SHP2. Indeed, studies have shown that expression of

catalytically inactive dominant negative SHP2 mutants results in increased gp130, JAK and STAT3 phosphorylation as well as gene induction (Lehmann *et al.*, 2003; Symes *et al.*, 1997). Further studies in AS-Ms could investigate the involvement of SHP2 and other possible inhibitory molecules in this inhibitory mechanism. Despite observing different responses in HUVECs and AS-Ms, AS-Ms may still represent a useful and tractable cell type to study in the context of endothelial inflammation. Indeed, it is likely that different inhibitory molecules are involved in the regulation of IL-6 and leptin signalling in different cell types, and studying AS-Ms as well as HUVECs would provide a greater understanding of all of these inhibitory mechanisms.

With repect to the SOCS3- and Epac1-dependent inhibition of IL-6/sIL-6α-stimulated ERK1,2 and STAT3 activation in HUVECs, further results from this study demonstrated a requirement for ERK1,2 activation in the inhibition of STAT3 activation. These results tie in well with recent findings from work carried out by Yarwood *et al.* (2008). These investigators have suggested that the C/EBP family of transcription factors link Epac1 activation to SOCS3 induction and since the C/EBP proteins have been shown to be substrates for ERK and RSK proteins (Ramji & Foka, 2002), an Epac1/ERK/C/EBP/SOCS3 pathway leading to the inhibition of IL-6/sIL-6α-stimulated STAT3 activation could be a possibility. However, this remains to be determined.

In conclusion, a potentially new pathway has been identified which inhibits cytokine receptor activation of ERK1,2 and STAT3 in endothelial cells. Additionally, the AS-M cell line has been further characterised in the context of cytokine and cAMP signalling. Overall, results from this study could contribute towards the identification of new molecular targets for the therapeutic treatment of chronic inflammatory diseases, such as atherosclersosis, and further examination of AS-Ms could uncover potentially new mechanisms of inhibition, or increase our knowledge of existing inhibitory mechanisms.

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