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Effect of Adenosine A_{2A} Receptor gene transfer on Nuclear Factor-κB-regulated inflammatory responses in vascular endothelial cells

Elaine Wenda Strong B.Sc. (Hons)

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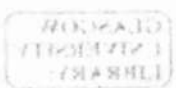
University of Glasgow



In memory of my father, Jim Ferrier

Who instilled in me the belief that anything is achievable with a little hard work and the confidence to carry it out.

Completed to program Dad.

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This thesis could not have been completed without the assistance and guidance from numerous people.

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Abbreviations

β_2AdAR	β_2-adrenergic receptors
A_1AR	A_1 Adenosine receptor
A_{2A}AR	A_{2A} Adenosine receptor
A_{2B}AR	A_{2B} Adenosine receptor
A_3AR	A_3 Adenosine receptor
AICAR	aminoimidazolecarboxaminoadenosineribonucleotide
AR	adenosine receptor
APS	ammonium persulphate
ARM	armadillo
AV	adenovirus
BCA	bichinchonic acid di-sodium salt
BIM	bisindolylmalemide 1
Blys/BAFF	B-lymphocyte stimulator/B-cell activating factor
BSA	bovine serum albumin
CaMKIV	Ca^{2+}/calmodulin kinase IV
CAM	cell adhesion molecule
CBP	CREB binding protein
CDK	cyclin-dependent kinase
cDNA	complimentary DNA
CKII	casein kinase II
CREB	cyclic AMP response element binding protein
COX-1	cyclooxygenase -1
COX-2	cyclooxygenase-2
DD	death domain
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EC	endothelial cells
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra-acetic acid

EGTA	ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunoabsorbant assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-related kinase
ET	endothelin
HEGF	epidermal growth factor
HEK293	human embryonic kidney 293 cells
FADD	Fas-associated death domain
hFGF-B	human fibroblast growth factor-B
FBS	foetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	gamma activation sequence
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRK	G-protein coupled receptor kinase
GST	glutathione S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDI	HDAC inhibitor
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesuphonic acid
HRP	horsradish peroxidase
HUVECs	human umbilical vein endothelial cells
IBB	importin-β-binding
ICAM	intracellular adhesion molecule
IFNγ	interferon-γ
IFNγR	interferon-γ receptor
IGF-1	insulin-like growth factor-1
IκB	inhibitory κB
IKK	IκB kinase
IL-1	interleukin-1

IL-1R	interleukin-1 receptor
Ig	immunoglobulin
IPTG	isopropyl-β-D-thioglalactopyranoside
IRAK	IL-1 receptor associated kinase
JAK1	Janus kinase 1
LBP	LPS binding protein
LMB	leptomycin B
LPS	lipopolysaccharide
MD-2	myeloid differentiation protein-2
MEK	mitogen-activated protein kinase/extracellular-signal related kinase kinase
MEKK	mitogen-activated protein kinase/extracellular-signal related kinase kinase kinase
mLDL	modified low-density lipoprotein
MOI	multiplicity of infection
MSK	mitogen- and stress-activated protein kinase
MyD88	Myeloid differentiation gene 88
NacCys	N-acetyl-$_L$-cysteine
NEMO	NFκB essential modulator
NES	nuclear export sequence
NFκB	nuclear factor κB
NK	natural killer
NLS	nuclear localisation sequence
NPC	nuclear pore complex
NO	nitric oxide
NOS	NO synthetase
NSAIDs	nonsteroidal anti-inflammatory drugs
NUP	nucleoporin
PAF	platelet-activating factor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDTC	pyrrolidinedithiocarbamate
PECAM	platelet-endothelial cell adhesion molecule

PFU	plaque forming units
PGs	prostaglandins
PGE_2	prostaglandin E_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
Pin-1	proline isomerase-1
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenlymethylsulphonyl fluoride
PP2	protein phosphatase 2A
PSGL	P-selectin glycoprotein ligand
RA	Rheumatoid arthritis
RANK	receptor activator of NFκB
Ran	Ras-related nuclear protein
RanGAP	Ran GTPase activating protein
RanBP	Ran binding protein
RHD	Rel homology domain
RIP	receptor interacting protein
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	synovial fibroblasts
SLP1	secretory leukoprotease inhibitor
SMC	smooth muscle cell
SOCS	suppressor of cytokine signalling
SODD	silencer of death domains
SRC	steroid receptor co-activator
STAT	signal transducer and activator of transcription
$TAF_{II}250$	TATA-box binding protein associated factor
TEMED	N,N,N`,N`-tetramethylethylenediamine
TIR	Toll-like/Interleukin-1 Receptor

TLR-4	Toll-like receptor-4
TM	transmembrane
TMB	3,3`5,5`-tetramethylbenzidine
TNFα	tumour necrosis factor-α
TNFR	TNFα receptor
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factor
TRIS	tris(hydroxymethyl)aminomethane
TXA_2	thromboxane
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WT	wild type

Pharmacological Names

CGS21680	2-*p*-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride
SAHA	Suberoylanilide hydroxamic acid
SB203580	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole
U0126	1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene
ZM241385	4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-α}{1,3,5}triazin-5-yl-amino]ethyl)phenol
8-pCPT-2'-O-Me-cAMP	8- (4- Chlorophenylthio)- 2'- O- methyladenosine-3', 5'- cyclic monophosphate

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Abstract

Inappropriate or prolonged inflammation contributes to the pathogenesis of many diseases including atherosclerosis, rheumatoid arthritis, sepsis, heart disease and cancer. It is widely accepted that the A_{2A} adenosine receptor (A_{2A}AR) is a critical non-redundant suppressor of inflammatory responses *in vivo*. Therefore understanding the mechanisms behind the suppression of inflammation would be beneficial in providing future drug targets for chronic inflammatory diseases. While the receptor is known to elevate intracellular cAMP levels, the molecular mechanisms underlying its inhibitory effects on defined pro-inflammatory signalling pathways remains obscure.

We have demonstrated that adenovirus-mediated human A_{2A}AR gene transfer into approximately 75% of virally-infected human umbilical vein endothelial cells (HUVECs) results in an expression level of 0.3-0.4 pmol/mg membrane protein as determined by ^{125}I-ZM241385 antagonist radioligand binding studies. A_{2A}AR-expression results in a constitutive suppression of TNFα-mediated induction of NFκB target genes such as cyclooxygenase-2 (COX-2) and the adhesion molecule E-selectin. To assess the functional significance of this phenomenon, the effects of A_{2A}AR gene transfer in HUVECs on the accumulation of three important gene products known to be controlled at least in part by NFκB, namely COX-2, E-selectin and vascular cell adhesion molecule –1 (VCAM-1) was determined. Characterisation of TNFα-mediated COX-2 induction revealed that while sustained induction (24hr) was solely dependent on the activation of p38 MAP kinase, earlier time-points at which induction was first detectable (8hr) were also sensitive to inhibition of the NFκB pathway. At this time-point, A_{2A}AR gene transfer alone was sufficient to reduce TNFα-mediated COX-2 induction compared with controls, whilst no effect of the A_{2A}AR was detectable at 24hr. Surprisingly, addition of the A_{2A}AR-selective agonist CGS21680 to A_{2A}AR-expressing HUVECs actually reversed the effect observed with receptor expression alone. Time-course experiments in control cells using elevated concentrations of the adenylyl cyclase activator forskolin demonstrated that

cAMP elevation was sufficient to promote the transient induction of COX-2 even in the absence of TNFα. However, in the presence of TNFα, exposure to different concentrations of forskolin exerted a biphasic effect, suppressing COX-2 induction at low concentrations while enhancing it at higher concentrations. These data suggest that the effects of A_{2A}AR expression with or without agonist on TNFα-mediated COX-2 induction can be accounted for completely by changes in cAMP. Comparative analysis with E-selectin revealed that induction of this gene was more straightforward, being sensitive to NFκB-inhibition alone with no contribution of any other signalling pathway being detectable. Moreover, while induction was suppressed by A_{2A}AR gene transfer, the suppression was more pronounced in the presence of CGS21680 and correlated with an ability of cAMP-elevating stimuli to block TNFα-mediated E-selection in control cells. Interestingly TNFα-mediated VCAM-1 induction was unaffected by either A_{2A}AR gene transfer or pre-treatment of control cells with cAMP-elevating agents. Taken as a whole, these data argue that the ability of the A_{2A}AR to modulate the expression patterns of defined κB-sensitive target genes in HUVECs can be explained completely by the ability of the receptor to positively couple to adenylyl cyclase and elevate intracellular cAMP. They also suggest that the net effects of A_{2A}AR gene transfer on specific κB-regulated genes will vary depending on the nature of their control by cAMP elevation.

Consistent with a suppression of NFκB signalling, A_{2A}AR expression specifically reduced the DNA binding capacity of p50/p65 heterodimers in response to TNFα stimulation. Subcellular fractionation studies demonstrated that this was the result of a dramatically reduced ability of TNFα to promote the time-dependent accumulation of p65 in the nuclei of A_{2A}AR-expressing cells compared with controls. Interestingly, the ability of TNFα to promote the IKK-dependent phosphorylation and subsequent degradation of IκBα and IκBε was unaffected. Thus, A_{2A}AR expression was either reducing nuclear importin-mediated nuclear import of p50/p65 heterodimers and/or accelerating the CRM-1-driven export of p50/p65/IκBα

complexes from the nucleus back into the cytoplasm. However, pre-treatment with a concentration of LMB that was effective in blocking CRM-1 mediated nuclear export of MEK-1 did not alter the inhibitory effect of A_{2A}AR gene transfer on TNFα-stimulated nuclear accumulation of p65. This suggests that the A_{2A}AR was selectively blocking nuclear import rather than accelerating nuclear export. This inhibitory effect of A_{2A}AR gene transfer did not appear to be due to a global inhibition of all nuclear import processes, as TNFα-stimulated p38 MAP kinase-mediated phosphorylation of the transcription factor ATF-2 was unaffected.

Chapter 1
Introduction

1.1 The Vascular Endothelium

The vascular endothelium lines the entire cardiovascular system and acts as a nonthrombogenic and selectively permeable barrier between the blood stream and extravascular space. Thus, the endothelium by its anatomical location is an enormous organ covering an area up to 7000m^2 in adults (Risau and Flamme, 1995), and possesses a unique and influential position during normal and pathophysiological processes.

1.1.1 The Structure of the Vascular Endothelium

The vascular endothelium forms a one cell thick covering on blood vessel walls and has a typical epithelial-like, cobblestone flat morphology. Developmentally, these cells are derived from the bone marrow, and in humans, begin to form blood vessels at day 21 of embryonic development. The structure of the cardiovascular system means that at different positions along the vascular tree, endothelial cells (ECs) have to cope with a wide range of physio-chemical conditions. In the human body there are a number of different EC phenotypes from certain areas of the body which have arisen in response to their specific environments. Although all vessels are subjected to laminar shear stress (Brooks et al., 2004), large arteries also have to cope with turbulent shear stress at branch points and pulsatile stress forces, whereas capillaries have to deal with changes in oxygen tension, toxin levels and in certain areas such as the skin, temperature fluctuations (Karlsson, 1985). While most endothelia form a selectively permeable barrier to the free exchange of blood components with the extravascular space, the degree of permeability is dependent on the specific EC site. At the blood-brain barrier permeability is minimal due to a high density of intercellular tight junctions, whereas some vascular beds have low levels of tight junctions. In environments where sampling or filtration occurs, such as glomeruli in the kidney and the circumventricular organs of the brain (Goss et al., 1986), gaps have been found between adjacent cells.

As well as displaying structural differences, different vascular beds also exhibit distinct biochemical and immunological properties. For example, venous ECs express more autocoid receptors and are able to induce the expression of adhesion molecules at a greater rate (Fisslthaler et al., 1999; Simionescu et al., 1982) than arterial ECs which typically generate greater levels of autocoids, including prostacyclin and nitric oxide (NO) (Fisslthaler et al., 1999). Despite this, ECs also have several common functions in normal physiological states including regulating blood flow and local coagulation status, maintenance of a local anti-inflammatory state and regulating the cell growth and survival required for angiogenesis (Sands and Palmer, 2005).

1.1.2 Endothelial Cell Signalling

The endothelium not only provides a structural barrier between the circulation and surrounding tissue, but ECs also secrete mediators that influence vascular hemodynamics in the physiologic state. ECs contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2), was well as vasoconstrictors including endothelin (ET) and platelet-activating factor (PAF). These chemically diverse compounds are not stored in intracellular granules. Rather, their major biologic effects are regulated by localisation of specific receptors on vascular cells, through their rapid metabolism, or at the level of gene transcription. NO is constitutively secreted by ECs but its production is modulated by a number of exogenous chemical and physical stimuli, whereas the other known mediators (PGI_2, ET and PAF) are synthesised primarily in response to changes in the external environment.

1.1.2.1 Nitric Oxide

Nitric oxide (NO) is a heterodiatomic free radical product generated through the oxidation of L-arginine to L-citrulline by NO synthetases (Venema et al., 1995). Of the three NO synthetases (NOSs), eNOS is constitutively active in ECs but is stimulated further by receptor-dependent agonists such as thrombin, adenosine

5'-diphosphate and muscarinic receptor agonists, and/or shear stress, that increase intracellular calcium and perturb plasma membrane phospholipid asymmetry (Awolesi et al., 1994, Topper et al 1996). EC-derived NO has several important effects on the vasculature, including the inhibition of platelet adhesion, activation, secretion and aggregation (Desjardins and Balligand, 2006). In addition NO also inhibits leukocyte adhesion (discussed in detail in section 1.2) to the endothelium (Kubes et al., 1991, De Caterina et al., 1995) and inhibits smooth muscle migration (Marks et al., 1995) and proliferation (Garg and Hassid, 1989). These latter effects have been shown to limit neointimal proliferation which occurs after vascular injury, and combined with the stimulatory effect of NO on EC migration and proliferation (Fukuo et al 1995) suggests that NO helps to sustain vascular reparative mechanisms.

1.1.2.2 Endothelins

Endothelins (ETs), potent vasodilators, are a family of 21-amino acid peptides produced by many cell types including ECs (Levin et al.,1996). ET-1 is formed from the inactive precursor preproendothelin-1 of ET-1, which is transcribed after stimulation by hypoxia, shear stress and ischaemia. ET-1 is released from ECs and binds to the abundant GPCR ET-A receptor on vascular smooth muscle cells, resulting in an increased intracellular calcium concentration which in turn increases vascular smooth muscle cell tone (Simonson and Dunn, 1990). NO has been shown to shorten the duration of these effects by accelerating the restoration of intracellular calcium to basal levels (Goligorsky et al., 1994). In states of endothelial dysfunction, such as atherosclerosis, in which concentrations of bioactive NO are reduced, the relatively unopposed actions of ET-1 promote vasoconstriction and smooth muscle proliferation (Lopez et al., 1990)

1.1.2.3 Prostacyclin (PGI_2)

Prostacyclin (PGI_2) is a vasoactive lipid compound synthesised by stimulated ECs and elicits smooth muscle relaxation by activating specific cell-surface Gs-

coupled GPCRs thereby elevating intracellular cyclic adenosine monophosphate (cAMP) levels (Mitchell and Warner, 2006). PGI_2 is not present in resting human ECs nor stored within the cell. Its synthesis is regulated by cyclooxygenase (COX) isoforms and prostaglandin isomerases from arachidonic acid (Grosser, 2006, Cines et al., 1998). Once synthesised PGI_2 acts on the IP receptor present on vascular smooth muscle cells, ECs and platelets (Coleman et al., 1994, Grosser, 2006), leading to vasodilation, reduced platelet aggregation and adhesion and reduced cholesterol uptake (Mitchell and Warner, 2006).

1.1.2.4 Reactive Oxygen Species

Oxidative stress is a state in which excess reactive oxygen species (ROS) overwhelm endogenous antioxidant systems. ROS have distinct functional effects on each cell type in the vasculature and can play both physiological and pathophysiological roles (Tanniuama and Griendling, 2003). One of the most important ROS in the vasculature is superoxide ($O_2^{\cdot-}$) which is formed by the univalent reduction of oxygen (Droge, 2002). Although $O_2^{\cdot-}$ can exert effects on vascular function, it is also pivotal in generating other reactive species including peroxygnitrite and hydrogen peroxide (H_2O_2) which in turn can react with reduced transition metals producing the highly reactive hydroxy radical ($^{\cdot}OH$) or become metabolised by myeloperoxidase to form hypochlorous acid. Virtually all types of vascular cells produce $O_2^{\cdot-}$ and H_2O_2 (Griendling et al., 2000).

Many functions of the endothelium are affected by ROS. These include impaired endothelial-dependent vasorelaxation due to an inactivation of $NO^{\cdot}$ by $O_2^{\cdot-}$ (Cai and Harrison 2000), endothelial apoptosis due to exposure to $O_2^{\cdot-}$ and H_2O_2 (Dimmeler and Zeiher, 2000), expression of adhesion molecules (Marui et al., 1993, Khan et al., 1996) and angiogenesis due to H_2O_2 induced proliferation and migration of ECs (Maulik and Das, 2002) and NAD(P)H oxidase regulation of VEGF expression (Ushio-Fukai et al., 2002).

1.1.2.5 Interaction between ECs and Blood Cells

In addition to the regulation of vascular tone, ECs also express cell surface-receptors that orchestrate the trafficking of circulating blood cells. These cell-associated molecules help direct the migration of leukocytes into specific organs under physiologic conditions and accelerate migration towards sites of inflammation (discussed in more detail in section 1.2).

1.1.3 The role of the Vascular Endothelium in Inflammation

Inflammation is characterised by the excessive infiltration of leukocytes from the blood into tissues following cellular injury. This movement is regulated by the sequential release of vasoactive and chemotactic mediators. Local vasodilation increases regional blood flow to the inflamed area and, together with an increase in microvascular permeability, results in the loss of fluid and plasma proteins into tissues. Under normal conditions this response is relatively short-lived and ultimately leads to the restoration of tissue structure and function. However prolonged inflammation can cease to be beneficial and contributes to the pathogenesis of many disease states such as atherosclerosis and rheumatoid arthritis (Karouzakis et al., 2006). Thus, the endothelium has been strongly implicated in the pathology of many inflammatory disease states including rheumatoid arthritis, atherosclerosis, systemic lupus erythromatosis, septic shock and transplant rejection (Anderson et al., 1995; D'Cruz, 1998; Gueler et al., 2004; Middleton et al., 2002; Zimmerman et al., 1999) due to the translocation of leukocytes into the extravasculature tissue. A brief description of the pathogenesis of atherosclerosis and rheumatoid arthritis are detailed below.

1.1.3.1 Atherosclerosis

Atherosclerosis is a slowly progressing inflammatory disease of the medium and large-sized arteries (Ross, 1999), resulting in the formation of fatty and fibrous lesions in the vessel wall. Initiation of atherosclerosis is characterised by the formation of modified low-density lipoprotein (mLDL), followed by the

subsequent activation of the endothelial layer, secretion of chemokines and the attraction of leukocytes. On migration through the endothelial layer, the recruited leukocytes differentiate into macrophages and scavenge the mLDL from the vessel wall, resulting in foam cell formation. This process is accompanied by inflammatory responses. Later stages involve the proliferation and migration of smooth muscle cells (SMCs) resulting in the formation of a fibrotic lesion with a fibrous cap of SMCs and an extracellular matrix that encloses a lipid-rich necrotic core. These lesions can become increasingly complex, with calcification, ulceration at the luminal surface and haemorrhage from small vessels that grow into the lesion from the media of the blood vessel wall (reviewed in Libby, 2002; Lusis, 2000). Although advanced lesions can grow sufficiently large enough to block blood flow, the most common clinical complication is an acute occlusion. This is due to the formation of a thrombus or blood clot associated with rupture or erosion of the lesion, resulting in myocardial infarction or stroke. The nuclear factor κB (NFκB) which regulates gene expression of many pro-inflammatory markers including selectins and adhesion molecules, has been implicated with a role in atherosclerosis pathophysiology (de Winther et al., 2005)

1.1.3.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with the destruction of affected joints. The inflamed synovium within the joints consist of diverse cell populations including B cells, T cells, macrophages and synovial fibroblasts (SF). The presence of these cells lead to the abnormal immune phenomena in the joint, namely chronic inflammation and synovial hyperplasia. All four populations of cells contribute in their own way to the promotion and perpetuation of the disease (Karouzakis et al., 2006). The immune cells interact with the SF to maintain inflammation within the joint via the production of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin-1 (IL-1). TNFα and IL-1 are secreted along with other cytokines by macrophages when they are in contact with T cells in the inflamed synovium.

Inhibition of TNFα via monoclonal antibodies such as Infliximab and Adalimumab and soluble receptors have been successfully used in the treatment of RA (Maini et al., 1999). In addition B cells also contribute to the pathogenesis of the disease by producing rheumatoid factors and autoantibodies (Takemura et al., 2001, Edwards et al., 2004).

1.1.4 Strategies to Reduce Inflammation

Various strategies to reduce inflammation have been reported and some are currently used clinically to treat chronic inflammatory diseases. These include antibodies against pro-inflammatory cytokines and administration of glucocorticoids (Adcock et al., 2004; Weaver, 2004).

1.1.4.1 Biological Therapy

Biological therapy selectively targets mediators believed to be involved in the pathogenesis of pro-inflammatory diseases such as RA (Arend et al., 1998; Taylor, 2001). The actions of these mediators are normally maintained in balance by a variety of anti-inflammatory and immunoregulatory cytokines. In diseases such as RA, an imbalance occurs favouring the pro-inflammatory cytokines, specifically Interleukin-1 (IL-1) and TNFα (Arend, 2001). IL-1 and TNFα regulate the expression of cell adhesion molecules and are able to increase the expression of various pro-inflammatory mediators including NO and prostaglandin E_2. There are several of these biological therapies that have been evaluated in patients with active RA, including anti-TNFα antibodies Infliximab and Adalimumab, the soluble TNF receptor Etanercept and the IL-1 receptor agonist Anakinra.

Clinical trials with Infliximab, a chimeric human-murine anti-TNF monoclonal antibody, and Adalimumab, the human anti-TNFα antibody, in patients with active RA have been evaluated. In these trials combination treatment of either antibody along side methotrexate showed a significant decease in disease

progression when compared to methotrexate treatment alone (Maini et al., 1999; Weinblatt et al., 2003). Both of these antibodies are used clinically in the treatment of RA.

Etanercept, a fusion protein containing the extracellular domain of the TNF receptor, has been evaluated as monotherapy and in combination with methotrexate in patients with active RA (Bathon et al., 2000; Moreland et al., 1999) and been recommended for use in RA treatment in both cases (Weaver, 2004).

Anakinra is an exogenous recombinant human IL-1 receptor agonist which differs from the non-glycosylated form of natural IL-1 receptor agonist in a terminal methionine (Weaver, 2004). Monotherapy and combination therapy with methotrexate, clinical trials of anakinra proved to reduce the rate of bone erosion of patients compared to methotrexate treatment alone (Bresnihan et al., 1998; Cohen et al., 2002; Jiang et al., 2000).

1.1.4.2 Glucocorticoids

Glucocorticoids, a class of steroid hormones, are widely used as anti-inflammatory and immunosuppressives drugs to treat inflammatory conditions, such as RA and asthma, and as adjunction therapy for conditions such as autoimmune diseases. Glucocorticoids have been shown to bind to cytoplasmic glucocorticoid receptors (GRs; GRα has been shown to be the most important) that translocate to the nucleus, where they bind to glucocorticoid response elements (GREs) in the promoter region of glucocorticoid-sensitive genes. This leads to the recruitment and activation of transcription coactivator molecules and the activation of genes encoding anti-inflammatory proteins such as secretory leukoprotease inhibitor (SLP1), β_2-adrenergic receptors and CD163, a cell-surface haemoglobin-complex receptor on macrophages. In spite of this ability to induce anti-inflammatory gene transcription, the major anti-inflammatory effects of glucocorticoids are through the repression of

inflammatory and immune genes. These inhibitory effects appear to be due largely to interactions between the activated monomeric GRα and transcription factors for inflammatory genes such as NFκB and AP-1 (Adcock et al., 2004; Karin, 1998). For example, glucocorticoids have been shown via the inhibition of NFκB, to reduce the activation of vascular endothelial cells, their expression of ligands for leukocyte adhesion molecules, and atherosclerosis. Monoclonal antibodies to vascular endothelial intercellular adhesion molecule-1 inhibited hypoxia-induced atherosclerosis in transgenic sickle mice (Okpala, 2006).

1.1.4.3 Other Potential Approaches

In addition to the NFκB pathway, cytokines and TLR agonists are also able to activate p38 MAP kinase pathway which control the transcription of AP-1-regulated genes. Resent studies have shown that inhibitors of these transcription factors are able to block inflammation by preventing the transcription of adhesion molecules and other pro-inflammatory genes, as outlined below:-

Inhibition of NFκB pathway by IKK inhibitors or the introduction of IKK complex-disrupting peptides (NEMO-binding domain peptides) have recently been reported. For example, a cell-permeable synthetic peptide corresponding to the IKKγ α-helical region, which prevents IKKγ from interacting with the IKKα/β complex, has been shown to block TNFα-induced NFκB activation (May et al., 2000; Paria et al., 2003), and a recent study of human cytomegalovirus-infected human umbilical vein endothelial cells (HUVECs) has shown that treatment with a specific IKKβ inhibitor, AS602868, also impaired HCMV-induced NFκB activity and subsequently prevented up-regulation of inflammatory marker proteins including cyclooxygenase–2 (Caposio et al., 2007).

In addition to the induction of cytokines and pro-inflammatory markers, p38 MAP kinase activity has been shown to have a key role in the pathogenesis of Crohn's disease (Hollenbach et al., 2004). There are many p38 MAP kinase

inhibitors currently in development and at various stages within clinical trials including AMG-548, BIRB-796, SCIO-469 and VX-702 (Dominguez et al., 2005; Fantini et al., 2006).

1.2 Leukocyte Translocation

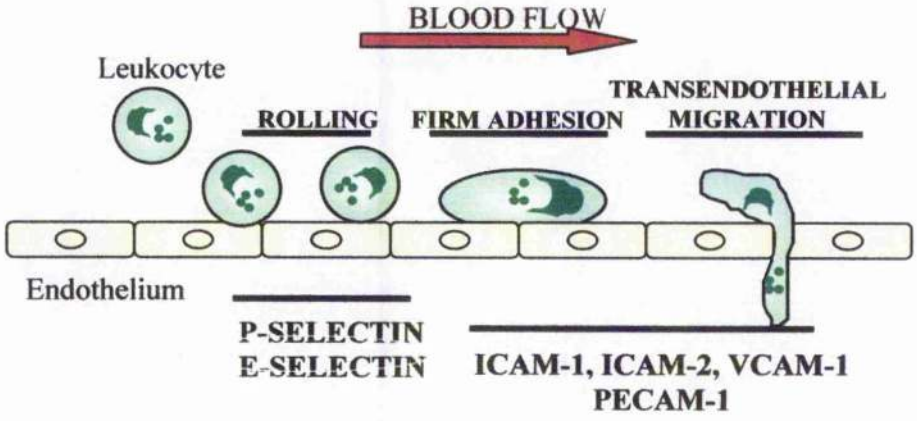
Leukocyte translocation into the extravasculature tissue occurs through a multistep process in which leukocytes interact with the endothelium in post capillary venules and is indicated by the presence of selectins, integrins and other pro-inflammatory markers. The three main steps involve initial recognition and recruitment of activated leukocytes, firm adhesion between leukocytes and the endothelium and finally migration, or diapedesis of leukocytes from the blood through to the underlying inflamed area (Figure 1.1). Activation of leukocytes and the endothelium is mediated via soluble inflammatory cytokines (Section1.3), such as tumour necrosis factor α (TNFα) and interferon γ (IFNγ) which are secreted by the surrounding cells upon recognition of different pathogenic stimuli by specific cell-surface receptors. Once activated, initial recruitment and rolling of the leukocytes over the endothelium is determined by the expression of selectins. For example, studies using knockout mice have shown that effective leukocyte recruitment requires the expression of two specific selectins (P- and E-selectin; Jung and Ley, 1999; Robinson et al., 1999). This emphasises the important role selectins play in leukocyte trafficking.

1.2.1 Selectins

The selectin family consists of three members E-, P- and L-selectin which all mediate rolling of leukocytes along the endothelium (Ley, 2001; Vestweber and Blanks, 1999). They range in size from 138-212 amino acids and have a conserved domain structure, consisting of an N-terminal lectin-like domain, a short transmembrane region and a short C-terminal tail (Ley 2003). The expression of each selectin is cell-type-specific. Thus, L-selectin is

Figure 1.1 Leukocyte Translocation

Different adhesion molecules are required for the translocation of leukocytes from the blood into the underlying site of injury. The left panel shows the selectins and their corresponding high affinity carbohydrate ligands which are involved in the initial recruitment and rolling of the leukocytes along the endothelium (Section 1.2.1). The right panel shows the predominance of cell adhesion molecules (CAMs) and integrin interactions involved in the arrest and subsequent translocation of leukocytes (Section 1.2.2-1.2.3).



constitutively expressed on leukocytes, while P-selectin is stored on α-granules of platelets and in Weibel-Palade bodies of endothelial cells and is rapidly transferred to the cell surface upon cellular activation. In contrast, E-selectin is exclusively expressed on endothelial cells only following exposure to pro-inflammatory stimuli (Ulbrich et al., 2003). All selectins bind the tetrasaccharide sialyl $Lewis_{(X)}$ ligand which is expressed on inflammatory target cells. An important molecule in leukocyte presentation is P-selectin glycoprotein ligand (PSGL). Interaction between PSGL and P-selectin is responsible for a major part in leukocyte rolling (Yang et al., 1999). Although it has been suggested that E-selectin interacts with PSGL, the identity of the true ligands for E-selectin is still unclear (Xia et al., 2002).

The interaction of selectins on leukocytes and the endothelium, in the presence of pro-inflammatory mediators, leads to the expression of other types of adhesion molecule that mediate the firm adhesion of the leukocyte to the endothelium. These are members of the cell adhesion molecule (CAM) family and integrin adhesion molecules.

1.2.2 Cell Adhesion Molecules (CAMs)

CAMs are transmembrane glycoproteins that are characterised by the presence of several extracellular Ig-like binding domains. Although there are many Ig-domain containing proteins, the ones important to leukocyte-endothelium interactions are interacellular adhesion molecule-1 and –2 (ICAM-1 and -2), vascular cell adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule (PECAM). ICAM-1 contains five immunoglobulin (Ig)-like domains and is expressed in response to pro-inflammatory signals. Once expressed at the surface, ICAM-1 is able to bind to its integrin binding partner $\alpha_L\beta_2$ which is expressed on infiltrating leukocytes. In contrast, ICAM-2 is constitutively expressed by endothelial cells and contains only two Ig-like domains. These domains share 35% sequence homology with the last two ligand binding domains of ICAM-1 (Lehmann et al., 2003) resulting in a degree

of redundancy between the two ligands. There are other members of the ICAM family (ICAM-3-5) of which ICAM-3 is known to be expressed at low levels on endothelial cells. However they are not thought to be involved in leukocyte interactions (Patey et al., 1996).

VCAM-1 is predominately expressed on endothelial cells and has two isoforms; a full-length protein containing seven Ig-like domains in the N-terminus and a smaller protein that does not possess domain 4 which results in reduced affinity for integrin. In a similar mechanism to ICAM-1, full-length VCAM-1 is expressed in response to pro-inflammatory stimuli and binds $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins expressed on infiltrating leukocytes. VCAM-1 ligand binding is essential for lymphocyte migration into inflamed tissue as it stimulates NADPH oxidase-dependent production of reactive oxygen species by endothelial cells (Matheny et al., 2000).

PECAM primary transcripts are also subject to alternative splice events which results in several isoforms that differ in domain number (Wang et al., 2003). All of these are constitutively expressed on endothelial cells, platelets, monocytes, neutrophils and specific subsets of T-lymphocytes (Ulbrich et al., 2003). It is thought that the number of isoforms available enables PECAM to interact with several substrates including proteoglycans (DeLisser et al., 1993) and integrins ($\alpha_V\beta_3$; Buckley et al., 1996).

1.2.3 Integrins

Integrins are non-covalently associated heterodimers containing one α- and β-subunits comprising large ligand binding domains and short cytoplasmic domains. The binding of integrins to associated ligands expressed on infiltrating leukocytes causes changes in the cell cytoskeleton via the cytoplasmic domain. 19 α- and 8 β-subunits have been identified in vertebrates which are reported to form at least 25 $\alpha\beta$ heterodimers (Takagi and Springer, 2002). Integrins involved in leukocyte-endothelium interactions bind to CAMs.

These include $\alpha_L\beta_2$ integrin which binds to both ICAM-1 and –2 to promote firm arrest of the leukocyte on the endothelium, and $\alpha_v\beta_3$ which binds to PECAM. $\alpha_4\beta_1$ and $\alpha_4\beta_7$ mediate either rolling or firm arrest of lymphocytes and monocytes via interaction with VCAM-1. This has led to the α_4 integrins being identified as governing mononuclear trafficking (Berlin et al., 1995).

1.2.4 Cyclooxygenase-2 (COX-2)

Prostaglandins (PGs) are synthesised in various tissues and cells and are considered of importance in the modulation of vascular homeostasis, inflammation, pain and angiogenisis (Dormond et al., 2001; Turini and DuBois, 2002). The major rate-limiting enzyme involved in their synthesis are the cyclooxygenases (COXs). The COX proteins are bifunctional enzymes containing a cyclooxygenase catalytic centre which adds 2 oxygen atoms to arachidonic acid in a bisoxygenation reaction to form the peroxide PGG_2, and a peroxidase active site which reduces PGG_2 to prostaglandin H_2 (PGH_2) (Smith et al., 1996a). PGH_2 is a common precursor for the synthesis of biologically active prostanoids including prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) and thromboxane (TXA_2). Of the two COX isoforms, COX-1 is constitutively expressed whereas COX-2 is highly induced by pro-inflammatory stimuli such as cytokines as well as growth factors such as transforming growth factor (TGF)-beta1. COX-2 has been shown to be a major source of prostacyclin which is produced in response to endogenous and exogenous signals to regulate vascular homeostasis in cardiac tissues and vascular endothelial cells (Adderley and Fitzgerald, 1999; Hao et al., 2002; Wu, 1998). This regulation is due to an alteration of the prostanoid ratio from a pro-thrombotic (high TXA_2/PGI_2) to an anti-thrombotic (high PGI_2/TXA_2) state (Caughey et al., 2001). It is important to note that this change in ratio is due to the increased concentration of PGI_2 concentration due to the induction of COX-2 not a decrease in COX-1 dependent TXA_2 concentration. Although selective COX-2 inhibitors have been shown to suppress unwanted inflammation in patients with rheumatoid arthritis and osteoarthritis (Crofford et al., 2000) with decreased

upper gastrointestinal side effects compared with nonsteroidal anti-inflammatory drugs (NSAIDs) that non-selectively block the binding to COX receptors (Crofford et al., 2000; Simon et al., 1999), up-regulation of COX-2 in the vascular endothelium may represent an important protective mechanism against vascular injury and insult. This corresponds with the reasons that the Merck COX-2 inhibitor *Vioxx* was taken off the market due to increase incidence of myocardial infarction and other cardiovascular related diseases (Mitchell and Warner, 2006).

1.3 Cytokines

The endothelium is capable of expressing a broad spectrum of pro-inflammatory (TNFα, IL-1, IL-6) and anti-inflammatory cytokines (IL-4, IL-10), including members of the TNF family, the interferons and the interleukins (Kofler et al., 2005). Cytokines are small soluble proteins which are secreted by one cell but are able to alter the behaviour or properties of itself or other cells. They are usually glycoproteins secreted by cells using classical secretory pathways and can be grouped into families depending on structure. In the majority of cases, expression patterns of different forms of cytokines or members of a cytokine family are only partially overlapping. This suggests a specific role for each family. This section will look at selected cytokines in greater detail.

1.3.1 Tumour Necrosis Factor α (TNFα)

TNFα is synthesised by macrophages and other cells in response to invasive stimuli such as bacterial toxins and inflammatory products. It is a homotrimer that is generated by the proteolytic cleavage of transmembrane TNFα by a metalloprotease TNFα-converting enzyme (Moss et al., 1997). Although first recognised as a soluble factor responsible for the necrosis of tumours in mice, it is now known to be part of a wider TNF superfamily, members of which are expressed throughout the vasculature. Each TNF family member binds to its associated receptor expressed on its target cells which mediates its effects. There are two receptors that are selective for TNFα, TNFR1 (75kDa) and

TNFR2 (55kDa) which are present on practically all cells except erythrocytes. These receptors share structural homology in the extracellular TNF-binding domains and exhibit similar binding affinity for TNFα, however they induce separate cytoplasmic signalling pathways following receptor ligand binding (Smith et al., 1990; Thoma et al., 1990).

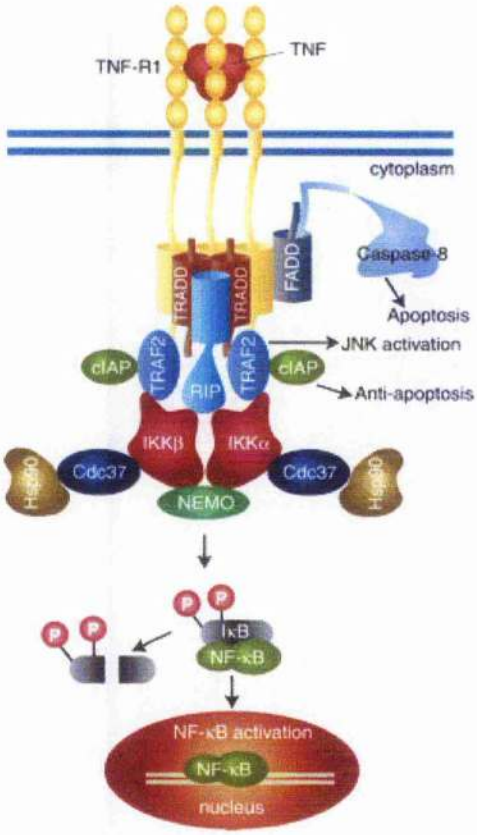
TNFR1 comprises of an N-terminal TNFα-binding domain, a short transmembrane region and a C-terminal intracellular death domain (DD). Upon TNFα binding, the receptor clusters with two other receptors to form a trimer which causes the release of the protein, silencer of death domains (SODD) (Chen and Goeddel, 2002). This exposes the DD of the receptor, allowing it to act as a scaffold for other DD-containing proteins including the adaptor protein, TNFα-receptor-associated death domain (TRADD). Once bound TRADD can then recruit additional adaptor proteins, receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) and Fas-associated death domain (FADD; Figure 1.2). These proteins are then able to recruit key enzymes to TNF-R1 that are responsible for initiating further downstream signalling events including IκB kinase (IKK; section 1.5) which results in the activation of the nuclear transcription factor κB (NFκB; section 1.5) (Dempsey et al., 2003).

1.3.2 Interferon γ (IFNγ)

The potent anti-viral properties of interferons were first identified due to their ability to interfere with viral replication (Isaacs and Lindenmann, 1987) since then it has been shown that they have several other roles including a role in microbial defence (Shtrichman and Samuel, 2001). The interferon family can be divided into two classes (I and II) with IFNγ being the sole member of the type II class. It is synthesised and released from cells of the immune system such as natural killer (NK) cells and T lymphocytes in response to other pro-inflammatory stimuli such as interleukin-12 (IL-12; Lammas et al., 2000) and TNFα. 17kDa in size, IFNγ functions as a dimer, binding to the IFNγ receptor (IFNγR) which is expressed on nearly all cell surfaces (Ramana et al., 2002).

Figure 1.2 Schematic of TNFα signalling

TNFα binding induces receptor trimerisation leasing to the release of SODD allowing the interaction of the receptor death domains. Receptor trimerisation generates a scaffold for the recruitment of adaptor proteins such as TRADD, TRAF2, RIP, and FADD. Recruitment of TRAF2 and RIP is necessary to activate the IKK complex leading to the phosphorylation-dependent polyubiquitination and subsequent degradation of IκB. This allows activated NFκB to translocate to the nucleus and activate transcription (section 1.4). (taken from Chen and Goeddel, 2002)



The IFNγR consists of two heterologous subunits (α- and β-). Upon receptor activation, Janus activated kinase 1 (JAK1) is recruited to the α- subunit whilst JAK2 is recruited to the β- subunit of the receptor (Shtrichman and Samuel, 2001). The binding of JAK 1 and 2 activates the JAK dimer which leads to the Tyr phosphorylation of signal transducers and activators of transcription (STAT) proteins. Once phosphorylated STAT1 undergoes homologous dimerisation and translocates to the nucleus where it binds to target genes containing the gamma activation sequence (GAS; consensus = $TTCN_3GAA$) initiating transcription of genes including iNOS and suppressor of cytokine signalling-1 (SOCS-1) (Samuel, 2001).

1.3.3 Lipopolysaccharide (LPS)

Although not a member of the cytokine family, LPS is a potent pro-inflammatory stimuli. A major component of the outer wall in Gram-negative bacteria, it can activate endothelial cells, monocytes and macrophages to stimulate the production of many pro-inflammatory cytokines, including TNFα, and IL-1,-6,-8 and –12 (Cohen, 2002). In the blood LPS is bound to LPS-binding protein (LBP) that transfers it to CD14, which can be either bound or soluble as endothelial and epithelial cells do not have membrane-bound CD14 but are still LPS-responsive (Pugin et al., 1994). The LPS-CD14 complex is then able to bind to the Toll-like receptor-4 (TLR-4)-myeloid differentiation protein-2 (MD-2) receptor complex thus initiating LPS responsive signalling pathways. TLR-4 is a member of the wider Toll-like receptor family, which were first characterised in *Drosphila*, and are important in mediating dorsal-ventral polarity in embryonic development (Morisato and Anderson, 1994) and in generating the anti-fungal response in *Drosphila* (Lemaitre et al., 1996). There are ten human TLRs which all share a common Toll-like/Interleukin-1 Receptor (TIR) motif which generates the downstream signalling from the TLRs. This motif is also common to the IL-1R family of receptors and as a result the downstream signalling events of the TLR and IL-1R share a common pathway (Imler and Hoffmann, 2001; Janeway and Medzhitov, 2002; Takeda et al., 2003)

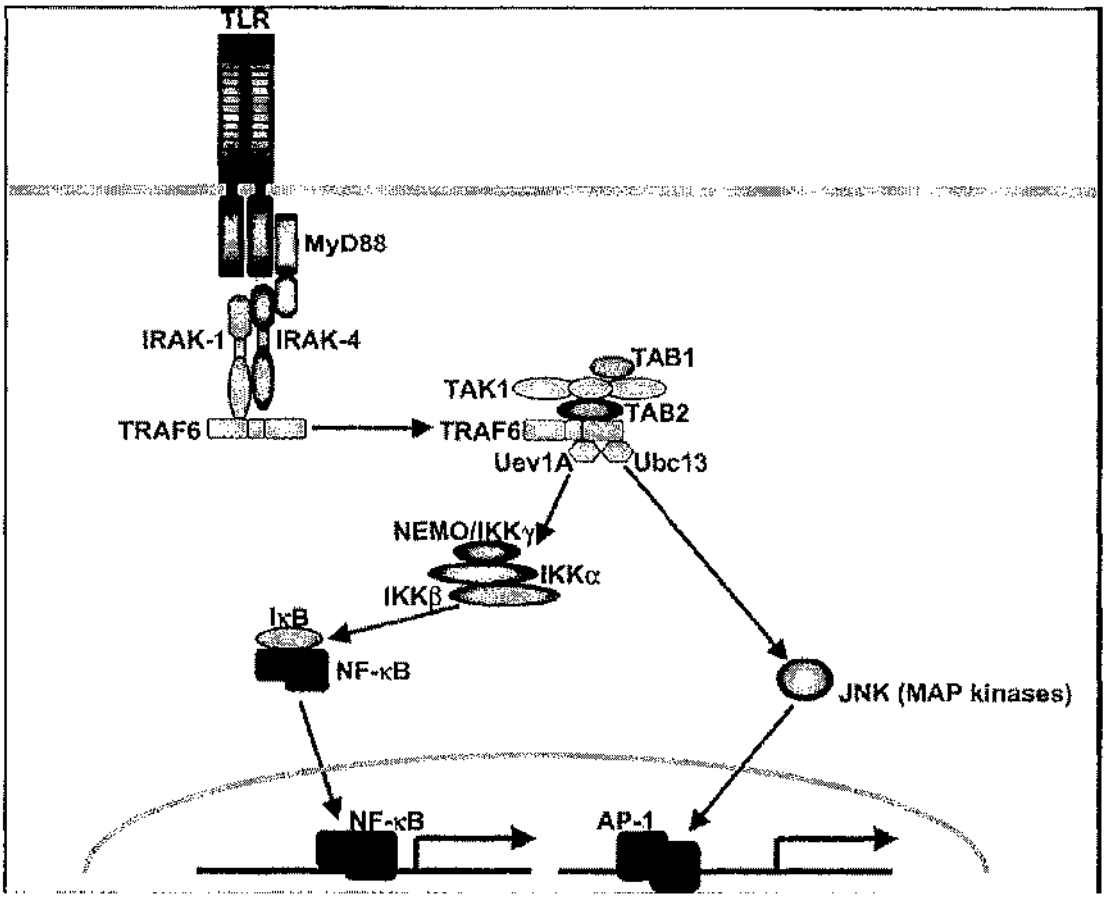
The binding of LPS to TLR-4 initiates the recruitment of a multi-protein complex that is assembled at the cytoplasmic tail of the receptor mediated by the TIR domain of the TLR (Figure 1.3; Takeda et al., 2003). Several different proteins have been identified as adaptor molecules for TLR-4. Myeloid differentiation gene 88 (MyD88) is a TIR containing TLR adaptor protein, which is recruited to and binds to the TIR domains of TLR-4 (Medzhitov et al., 1998; Wesche et al., 1997). It has also been reported that a MyD88-adaptor like protein Mal/TIRAP can also bind to the TIR domain of TLR-4 (Fitzgerald et al., 2001; Horng et al., 2001). Following the recruitment of MyD88 to TLR-4, IL-1 receptor associated kinase (IRAK) is recruited to the receptor via an interaction between the DD present on both proteins, where it becomes phosphorylated by IRAK-4 (Suzuki et al., 2002). There are four reported members of the IRAK family, IRAK-1, IRAK-2, IRAK-4, IRAK-M all of which contain a conserved DD and kinase domain (Li et al., 2002). It has been reported that each kinase interacts with TLR-4 through a variety of upstream adapter molecules which may explain the non-specific nature of these interactions (Janssens and Beyaert, 2003). IRAK-4 has been reported to have the most significant interaction as indicated by the lack of inflammatory response to LPS or IL-1 in IRAK-4 knockout mice (Suzuki et al., 2002). Activated IRAK is then able to interact with TRAF6 leading to the downstream activation of NFκB and JNK. Therefore activation of TLR-4 signalling by LPS leads to the transcription of many pro-inflammatory genes including adhesion molecules and COX-2.

1.4 Nuclear Factor κB (NFκB)

NFκB was first identified in 1986 by Sen and Baltimore, as a constitutively active nuclear factor binding to a site in the kappa light-chain Ig enhancer in murine B lymphocytes (Sen and Baltimore, 1986). Since then it has been identified as a transcription factor existing in an inactive state in the cytoplasm of most cells (Liou and Baltimore, 1993).

Figure 1.3 Schematic of LPS signalling via TLR4

Activation of the TLR-4 receptor complex leads to the recruitment of MyD88 via the TIR domain. The resulting phosphorylation of IRAK by IRAK-4 enables the recruitment of TRAF6 to the complex. Upon its activation, TRAF6 can interact with the TAK1/TAB1/TAB2 complex that also contains Uev1A and Ubc13. This leads to the polyubiquitination of TRAF6 and TAK1 activating downstream signalling to IKK and JNK. (Taken from Takeda and Akira, 2004)



It is now known that NFκB plays a central role in controlling gene expression of proteins involved in cell survival and pro-inflammatory responses. Activated by a range of stimuli including pro-inflammatory cytokines, growth factors, DNA-damaging agents and viral proteins (Ghosh et al., 1998), NFκB regulates a wide variety of target genes that include cytokines such as TNFα and IL-6, chemokines (e.g. IL-8, MCP-1), adhesion molecules (e.g. ICAM, VCAM-1 and E.selectin) and inducible effector enzymes such as COX-2 (Tanabe and Tohnai, 2002).

1.4.1 Overview of NFκB Signalling Pathway.

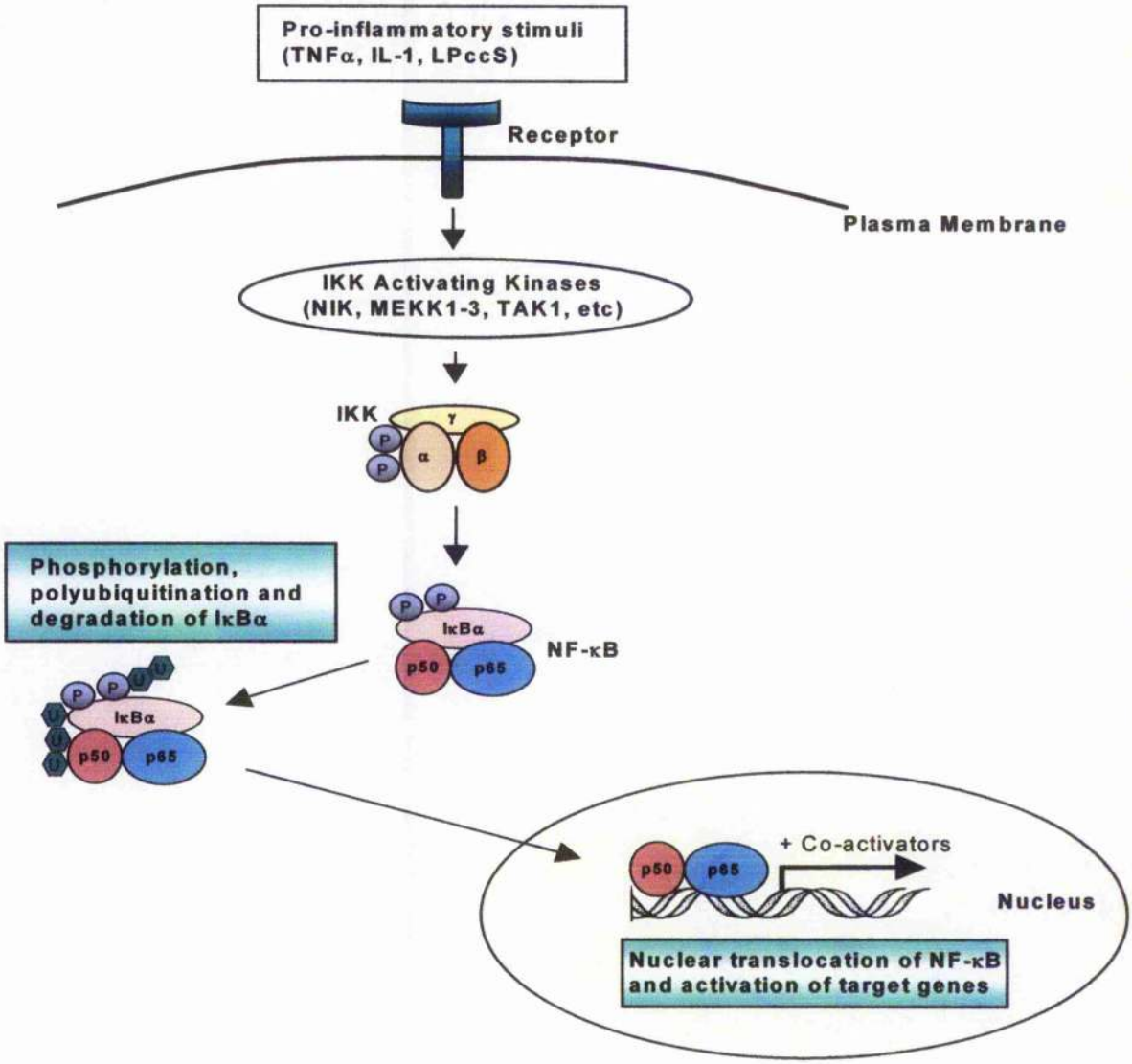
NFκB is localised in the cytoplasm of unstimulated cells, in an inactive state bound to a member of the IκB family of inhibitory proteins (Ghosh et al., 1998). The degradation of this inhibitor upon signal-induced activation has been regarded as the conventional model by which NFκB is activated (Baeuerle and Baltimore, 1996). This exposes the nuclear localisation sequence (NLS) of NFκB thereby allowing translocation to the nucleus to activate transcription. Many aspects of the signalling pathway that leads to the degradation of IκB family members have been described. Briefly, stimuli-specific signalling pathways converge on the IκB kinase (IKK) which is a multi protein complex comprising IKKα/1, IKKβ/2 and IKKγ/NFκB-essential modulator (NEMO). This specifically phosphorylates IκB at particular residues that prime IκB for Lys 48-polyubiquitination. Polyubiquitination targets IκB for proteolytic degradation by the 26S proteosome unmasking the NLS on NFκB enabling subsequent nuclear translocation and gene transcription (Figure 1.4). In reality the NFκB signalling pathway is far more complex involving multiple protein-protein interactions thus the following sections will examine this pathway in greater detail.

1.4.2 IκB Kinase (IKK)

The seminal event in the activation of NFκB is the phosphorylation of IκBs which is mediated by IκB Kinase (IKK). IKK is a 700-900kDa complex

Figure 1.4 Schematic of the classical NFκB signalling pathway

The detection of pro-inflammatory mediators at their specific cell surface receptors mediates the activation of IKK kinases leading to the phosphorylation of the IKK complex. This in turn results in the phosphorylation and polyubiquitination of IκB leading to the subsequent degradation. Thus allowing the activated NFB to translocate to the nucleus and initiate transcription of its target genes.



comprising several proteins of which the main three subunits are, IKKα (IKK1), IKKβ (IKK2) and the regulatory subunit NFκB essential modulator (NEMO) also known as IKKγ (Karin and Delhase, 2000). IKKα and IKKβ are catalytic subunits sharing 52% overall sequence homology and 65% identity within their catalytic domains. In contrast, IKKγ has no known kinase activity but contains the helix-loop-helix and leucine zipper motifs that are known to be involved in protein-protein interactions (see later; Karin and Delhase, 2000).

Gene targeting experiments have clearly demonstrated that the IKKβ and γ subunits of IKK are required for NFκB activation by all known pro-inflammatory stimuli, including LPS, TNF, double-stranded RNA, negative strand RNA viruses, IL-1 and antigens (Chu et al., 2000; Li et al., 1999; Makris et al., 2000; Tanaka et al., 1999). All of these stimuli lead to IKK activation which kinetics and magnitude are directly related to those of NFκB activation, although how they all converge to activate a single signalling enzyme still needs to be elucidated.

For example in the TNFα signalling pathway, binding of TNFα to TNFR1 recruits TRADD and TRAF2 causing the subsequent recruitment of the IKK complex to the receptor, where RIP1 can activate IKK1 via MEKK (Section 1.3.1; Devin et al., 2000; Hsu et al., 1996; Kelliher et al., 1998). The activity of RIP1 is not due to its protein kinase activity but due to Lys 63 polyubiquitination on Lys 377 allowing RIP to bind to IKKγ via its Leu zipper region (Chen, 2005; Ea et al., 2006). In addition, RIP1 is required for IKK activation by multiple pathways including the Toll-like receptors (TLR) and DNA damage pathways (Devin et al., 2003; Meylan et al., 2004) suggesting a common convergence point for NFκB activation.

The integration of the many upstream signals leads to the phosphorylation of conserved serine residues present in the activation loops of IKKα (S176, S180)

and IKKβ (S177, S181; Mercurio et al., 1997). Studies have shown that although cytokines such as TNFα result in phosphorylation of IKKα and IKKβ, IKKβ is predominantly required for NFκB activation by most pro-inflammatory stimuli (Li et al., 1999; Tanaka et al., 1999). However it is important to note a degree of specificity, since IKKα is required for IKK activation in response to RANK (receptor activator of NFκB) and the B-lymphocyte stimulator Blys/BAFF (B-lymphocyte stimulator/B-cell activating factor) (Cao et al., 2001; Claudio et al., 2002). Therefore, although certain signals can activate the kinase complex via IKKα, the majority of NFκB inducers target the IKKβ subunit. This complex regulation of IKK activity by upstream interactions ultimately results in the phosphorylation of the IκB family proteins.

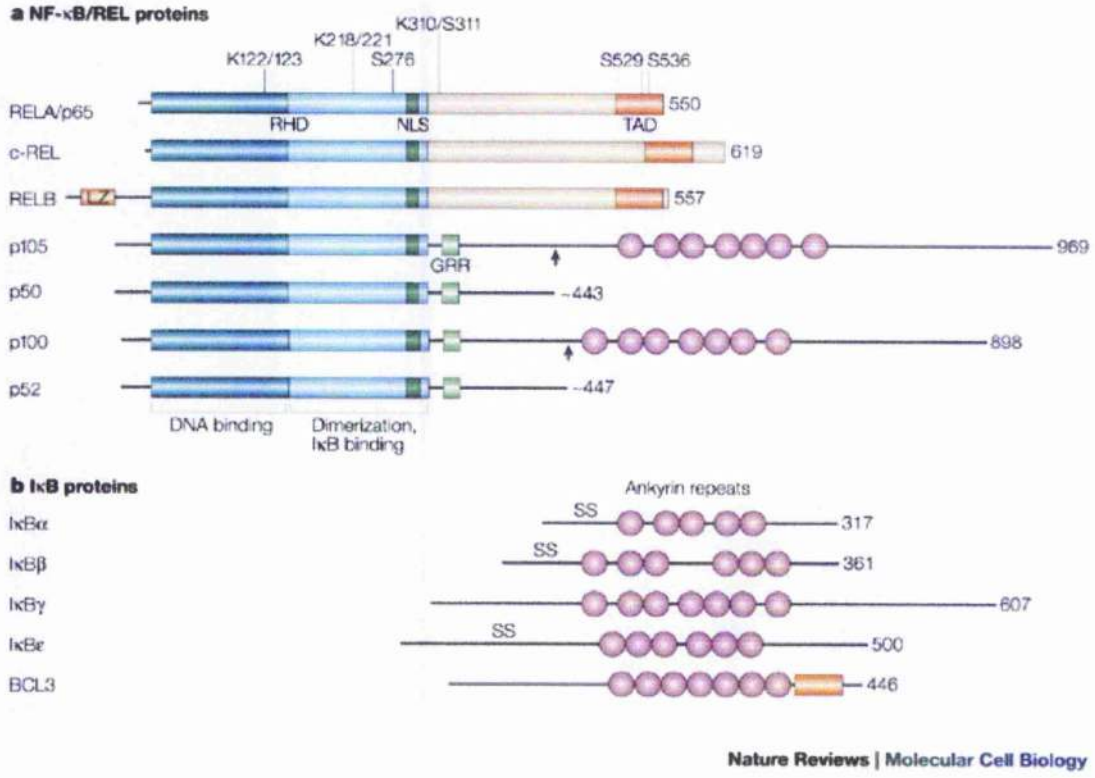
1.4.3 IκB Proteins

The IκB family comprises of eight members: IκBα, δ, β, γ, ε, ζ, R and bcl-3 (Ghosh et al., 1998; Ray et al., 1995; Yamazaki et al., 2001). IκBδ and IκBζ are the C-terminal products of p100 and p105 proteolytic processing, forming p50 and p52 respectively. The IκBs are characterised by the presence of multiple ankyrin repeats, protein–protein interaction domains responsible for interactions NFκB via their Rel homology domain (RHD) (Lux et al., 1990). Each IκB protein binds specifically to particular NFκB subunits. For example IκBα and β preferentially bind complexes containing p65 and c-Rel, while IκBγ binds to p50 and p52 homodimers (Thompson et al., 1995; Whiteside et al., 1997) and p100 and p105 precursors complex with p50, p65 and c-Rel in the cytoplasm. IκB members, bcl-3 and IκBζ are unique however as both are primarily expressed within the nucleus. Bcl-3 degradation causes p52-dependent repression of transcription (Bours et al., 1993; Viatour et al., 2003), while IκBζ retains NFκB proteins in the nucleus instead of the cytoplasm (Yamazaki et al., 2001).

Of the eight IκB proteins, only IκBα, IκBβ and IκBε are currently known to be involved in the signal-induced activation of NFκB (Figure 1.5). IκBα has been

Figure 1.5 NFκB and IκB Family Proteins

The mammalian NFκB family consists of five different members which all share a highly conserved Rel-homology domain (RHD). p65, c-Rel, and RelB also contain a transactivation domain necessary for the activation of transcription. p50 and p52 are cleaved from larger precursors p105 and p100 respectively (cleavage points indicated by ↑; section 1.4.4). The C-terminal products of this cleavage are IκBδ and IκBζ respectively which are two members of the wider IκB family. There are eight members in the IκB family which all contain ankyrin repeat regions required for interactions with NFκB (Section 1.4.3). Sites of phosphorylation (S) and acetylation (K) are indicated (taken from Chen and Greene, 2004)



the most extensively studied due to its ability to be rapidly degraded in response to most NFκB-activating stimuli. It is a 37kDa protein consisting of three domains, a 70 amino acid N-terminus containing a signal response domain, a central protease-resistant 250 amino acid domain consisting of six ARD repeats and a final 42 amino acid C-terminus comprising of PEST-rich (proline, glutamic acid, serine, theronine-rich) sequences that confer rapid basal IκBα turnover (Brown et al., 1997; Kroll et al., 1997; Lin et al., 1996). It has been suggested that the ankyrin repeats on IκBα not only prevent nuclear translocation of NFκB by masking its nuclear localisation sequence (NLS) but also inhibit the ability of NFκB to bind DNA (Beg et al., 1992; DiDonato et al., 1997; Henkel et al., 1992). In addition, IκBα also contains a leucine rich nuclear export sequence (NES) within the last ankyrin repeat which is recognised by the nuclear export protein CRM1, and is believed to actively export IκBα/NFκB complexes out to the cytoplasm thereby restoring the pre-induction state of the complexes (Arenzana-Seisdedos et al., 1997). To aid this restoration of the pre-induction state, transcription of IκBα is NFκB-dependent. Consequently, newly synthesised IκBα is able to enter the nucleus and sequester NFκB from κB-dependent promoters. This inhibits further NFκB transcription by facilitating export back into the cytoplasm via the NES on IκBα (Arenzana-Seisdedos et al., 1995). The mechanisms of nuclear trafficking will be discussed in more detail in section 1.5.

The degradation of IκB proteins occurs via a process known as polyubiquitination. Ubiquitin is a highly conserved 76 amino acid protein that is added to target proteins in three enzymatic stages (Pickart, 2004). Initially the C-terminal Gly residue of ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. In the second step, the activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2 or Ubc) on a specific Cys residue, forming an E2-Ub thioester. Finally, ubiquitin is attached to specific Lys residues on the target protein via an isopeptide bond mediated by a

ubiquitin-ligase, E3. Ubiquitin contains seven Lys residues that can be attached to other ubiquitins by the above pathway to form a poly-ubiquitin chain. Poly-ubiquitination, typically linked via Lys 48, targets the protein for protesomal degradation by the 26S proteasome (Nandi et al., 2006). Site-directed mutagenesis of lysine residues on IκBα identified K21 and K22 as the amino acids responsible for the covalent attachment of ubiquitin (Baldi et al., 1996; Rodriguez et al., 1996; Scherer et al., 1995). The formation of Lys 48-polyubiquitinated forms of IκBα has been shown to rely on the prior phosphorylation of serine 32 and serine 36 as mutagenesis of these sites prevented phosphorylation or ubiquitination whilst the K21R/K22R mutant was able to undergo signal-induced phosphorylation but not ubiquitination. Consequently neither mutant could be degraded preventing NFκB activation (Rodriguez et al., 1996).

1.4.4 NFκB

NFκB transcription factors are a family of structurally related and evolutionarily conserved proteins that form either homo- or hetero-dimers (Ghosh et al., 1998). All five mammalian NFκB subunits, Rel (c-Rel), p65 (RelA), RelB, NFκB-1 (p50 and its precursor p105) and NFκB-2 (p52 and its precursor p100) contain a highly conserved 300 amino acid long N-terminal Rel homology domain (RHD) responsible for DNA-binding, dimerisation and association with the IκB inhibitory proteins (Figure 1.5; reviewed in Ghosh et al., 1998). NFκB subunits can form various dimers, p65, Rel (c-Rel), and RelB contain at least one transcriptional activation domain in their C-terminus (Blair et al., 1994; Ryseck et al., 1992; Schmitz et al., 1994), and they can therefore form transcription-activating dimers with each other and with p50 or p52. p50 and p52 proteins lack the transcription activation domain, and the homodimers they form are mostly suppressors of gene expression (Zhong et al., 2002). However the classical, best-described form is composed of p50:p65 (Baeuerle and Baltimore, 1996; Thanos and Maniatis, 1995; Verma et al., 1995) and is found in virtually all cell types bound to IκBα (Baeuerle and Baltimore, 1996).

Following stimulation and subsequent degradation of IκBα, NFκB is able to rapidly translocate to the nucleus where it binds to a κB-binding motif conforming to the sequence, 5`GGGACTTTCC3` (Kunsch et al., 1992; Parry and Mackman, 1994). It has been shown that p50 will bind to the conserved 5` end, while p65 binds to the less conserved 3` end (Urban and Baeuerle, 1991). Other types of NFκB dimers bind preferentially to different DNA sequence elements, contributing to the specificity of target genes regulated by this family of transcription factors.

Crystal structures of NFκB bound to DNA have been solved for p50, p52, p65 homodimers and p50/p65 heterodimers and have indicated that the RHD is organised into three distinct sub-domains (Chen et al., 1998; Ghosh et al., 1995; Muller et al., 1995). The 180 amino acid N-terminal folds into an Ig-like domain connected via a short, 100 amino acid long, linker region to a second Ig-like domain of approximately 100 amino acids in length. The N-terminal Ig-like domain is involved in DNA recognition while the C-terminal Ig-like domain is involved in dimerisation. It was also found that fourteen residues from each subunit participate in dimer formation. The C-terminal Ig-like domain also contains the 13 amino acid nuclear localisation sequence (NLS) required for nuclear translocation (Birbach et al., 2002).

1.4.5 Regulation of NFκB by Post-translational Modifications

In addition to regulation by IκB, the localisation and transcriptional activity of p65 is regulated by phosphorylation-dependent acetylation (Chen et al., 2002). Acetylation is a common post-translational modification which was first associated with the regulation of histone packaging into chromatin fibres (Gray and Teh, 2001; Taunton et al., 1996). Acetylated histones are associated with transcriptionally active segments of chromatin, whereas deacetylated histones are present in transcriptionally inactive segments (Legube and Trouche, 2003; Marmorstein and Roth, 2001). Further studies indicated a range of transcriptionally co-activating proteins that contained histone acetyltransferase

(HAT) activity including CBP-associated factor (PCAF), p300/CREB binding protein (p300/CBP), TATA-box binding protein associated factor ($TAF_{II}250$) and steroid receptor co-activator (SRC-1) (Marmorstein and Roth, 2001). Subsequently proteins were also identified which contain histone deacetylase activity, termed 'HDACs', and HDAC3 has been found to be important for p65 deacetylation both invivo and invitro (Chen et al., 2001; Kiernan et al., 2003). Three main acetylation sites identified within p65 are lysines 218, 221 and 310 (Chen et al., 2002). The modification of these sites regulates distinct biological processes. For example, acetylation at K221 enhances DNA binding to p65 and impaires its assembly with IκBα, whereas acetylation of K310 is required for full transcriptional activity of p65 but modification of this site does not effect DNA binding or IκBα assembly (Chen et al., 2002). In conjunction, acetylation of two other sites K122 and K123, promotes post-induction removal of p65 from DNA and facilitates its translocation out of the nucleus (Kiernan et al., 2003). In contrast HDAC inhibitors (HDI) are also able to induce p65 acetylation (Chen et al., 2005; Xu et al., 2005) via a PI-3K/PKB dependent process (Mayo et al., 2003). It was recently reported by Liu et al., (2006) that treatment with the HDI, Suberoylanilide hydroxamic acid (SAHA), activated NFκB transcription via two separate mechanisms; the first via the de-repression of HDAC-1-mediated repression of p65, preserving the acetylation at K310 promoting NFκB transcription. The second mechanism involved the phosphorylation of PKB on S437 which transcriptionally up-regulated NFκB through its enhanced acetylation via an as yet undetermined mechanism.

Phosphorylation of p65 itself occurs at different phosphorylation sites, including S276, S529, S536 that are targeted by several upstream kinases including mitogen- and stress-activated protein kinase (MSK1; S276), protein kinase A catalytic subunit (PKA_C; S276), casein kinase II (CKII; S529), IKK (S536), protein kinase Cζ (PKCζ; RHD) and Ca^{2+}/calmodulin kinase IV (CaMKIV; c-terminus) (Vermeulen et al., 2002). Phosphorylation status is also determined by the activity of phosphatases such as protein phosphatase 2A (PP2A). PP2A

is associated with p65 in un-stimulated melanocytes and can dephosphorylate p65 following stimulation (Yang et al., 2001). Phosphoryiated p65 can then enter the nucleus where the specific phosphorylation pattern directs the interaction of NFκB with either p300/CBP or HDACs (Ashburner et al., 2001; Chen et al., 2002; Kiernan et al., 2003).

In conclusion, NFκB is an essential transcription factor required for the effective transcription of pro-inflammatory genes. Up-regulation of these genes increases surface expression of proteins which direct the recruitment of neutrophils, monocytes and macrophages to sites of inflammation. It responds to a diverse array of initial pathogenic stimuli via the integration of signals at the IKK complex leading to the degradation of IκB and the subsequent nuclear translocation of NFκB. Post-translational modifications such as phosphorylation and acetylation further regulate NFκB activity.

1.5 Mechanisms of Nuclear Import and Export of NFκB

The nucleus is the defining feature of the eukaryotic cell. The nuclear envelope divides the cell into two compartments between which there is a major interchange of proteins, nucleic acids and small molecules. To mediate the trafficking of molecules between the two compartments, the nuclear envelope contains nuclear pore complexes (NPCs). Small molecules are able to passively diffuse through these complexes whilst trafficking of molecules larger than 25nm in diameter is regulated by specific nuclear import and export systems. This section will examine the NPC followed by the nuclear import and export mechanisms utilised by activated NFκB.

1.5.1 Nuclear Pore Complex (NPC).

The NPC is a large 125 MDa multi-protein structure that completely spans the nuclear envelope, extending into both the cytoplasm and the nucleoplasm to a length of about 200nm (Reichelt et al., 1990; Stoffler et al., 1999). The pore has a total diameter of 25nm with a 9nm aqueous channel in the centre through

which passive diffusion can occur (Bonner, 1978; Paine et al., 1975), while proteins larger than 25nm require active transport to facilitate their trafficking through the pore (Gasiorowski and Dean, 2003). The NPC is composed of nucleoporins (NUPs) of which there are an estimated 30-50 NUPs present within eukaryotic NPCs. Although many of these NUPs are functionally conserved from yeast to mammals (Feldherr, 1962; Reichelt et al., 1990), there are many which are species-specific (Reichelt et al., 1990). Several NUPs have been found to reside on both sides of the pore whereas others are positioned either at the central core or asymmetrically on one side or the other (Stoffler et al., 1999). A known subset of NUPs has been found to contain the amino acid repeats FXFG or GLFG, which are believed to mediate interactions with transport receptors and act as docking sites for importins (Bonner, 1978; Singh et al., 1999). Thus they are implicated in facilitating movement across the NPC.

1.5.2 Nuclear Import of NFκB

Three essential proteins have been discovered to mediate nuclear import: importin α, which recognises cargo proteins with the classical NLS (KKKK) (Kalderon et al., 1984; Lanford and Butel, 1984), importin-β, which binds and ferries the complex into the nucleus, and the small regulatory GTPase Ran. Specifically when then α/β/NLS cargo complex reaches the far side of the pore, nuclear Ran-GTP binds to importin-β and dissociates the complex, thus completing import (Goldfarb et al., 2004; Harel and Forbes, 2004).

1.5.2.1 Importin-α

Importin-α proteins are composed of a flexible N-terminal importin-β-binding (IBB) domain and a highly structured domain comprised of ten tandem armadillo (ARM) repeats (Conti et al., 1998; Fontes et al., 2000; Herold et al., 1998). The helical ARM repeats assemble into a twisted, slightly arced structure, with the inner curve housing the cNLS-binding groove. ARM repeats 2-4 comprises the N-terminal NLS binding site and ARM repeats 7-9 the C-terminal NLS binding site. The flexible IBB domain acts as a competitive inhibitor regulating binding to

the cNLS-binding groove by interacting either in *trans* with importin β, targeting the complex to the NPC for translocation, or in *cis* with the cNLS-binding groove. This autoinhibitory interaction is not particularly strong as cNLS cargos can still bind to importin-α in the absence of importin-β. It is not known whether importin-α interacts first with importin-β to relieve the autoinhibition of the NLS-binging groove and then interact with the cNLS cargo, or the cNLS cargo binds to the NLS-binding groove first, thus liberating the autoinhibitory IBB domain to interact with importin-β (Goldfarb et al., 2004). It is possible that the order of binding varies from event to event depending on the particular cNLS cargo and the effective concentrations of the relevant factors.

Of the six importin-α family members identified in humans, importin-α3 has been shown to bind to the cNLSs reported on p50 and p65 (Blank et al., 1991; Fagerlund et al., 2005; Gilmore and Temin, 1988) following TNFα-mediated NFκB activation (Fagerlund et al., 2005). Importin-α3 is able to use either of its N- or C-terminal binding sites for binding different nucleus-targeted proteins (Melen et al., 2003). In a recent study it was postulated that p50 and p65 NLSs bound simultaneously to the different NLS binding sites of the same importin-α molecule possibly stabilising the complex during nuclear import (Fagerlund et al., 2005).

1.5.2.2 Importin-β

The concentration of importin-β within the cell has been estimated at 3μM (Kutay et al., 1997). Initial mutational analysis indicated that importin-β consisted of three domains, an N-terminal Ran-GTP binding domain, a middle zone that interacts with the nuclear pore and a C-terminal importin-α binding domain (Chi and Adam, 1997). However following crystal structure analysis, it emerged that importin-β consists entirely of 19 HEAT repeats, with each HEAT repeat containing two α-helices, A and B, joined by a loop. In addition, the protein coils into a short superhelix, with extensive interaction surfaces both on

the inside and the outside of the superhelix (Harel and Forbes, 2004). From the various proteins and protein fragments which have been co-crystallised with importin-β it has been shown that each protein utilises different binding sites and modes of interaction (as reviewed in Bednenko et al., 2003; Stewart, 2003). It appears that the spring-like superheiical importin-β has a large degree of flexibility (Stewart, 2003) which it utilises to recognise its many partners. For example, in some cases several importin-β HEAT repeats open up to accommodate the bound protein, whilst in other cases regions of the superhelix wrap around the chosen partner.

Importin-β mediates the interactions with the NPC that drive translocation. Importin-β has been shown to bind GLFG or FXFG repeat domains of several nucleoporins *in vitro* (Kraemer et al., 1995; Radu et al., 1995; Rexach and Blobel, 1995). However, invivo and functional significance of these interactions is controversial (Kau et al., 2004). Also the interaction with the channel of the NPC is as yet unclear.

1.5.2.3 Ran-GTP/GDP Gradient.

Ran, the Ras-related nuclear protein (Bischoff and Ponstingl, 1991; Drivas et al., 1990) is a key component of the import reaction and belongs to a class of small GTP binding proteins that cycle between a GTP- and a GDP-bound state. Although Ran has a very low intrinsic GTP hydrolysis rate, the presence of Ran GTPase activating protein 1 (RanGAP1) in the cytoplasm in conjunction with Ran binding protein 1 (RanBP1) on the cytoplasmic face of the NPC speeds up this GTPase activity. This results in a much higher concentration of RanGDP than RanGTP in the cytoplasm. Conversion of RanGDP to RanGTP occurs by exchanging the entire nucleotide and is catalysed by the principle mammalian guanine nucleotide exchange factor RCC1 (Bischoff and Ponstingl, 1991). RCC1 is a nuclear, chromatin-bound protein (Ohtsubo et al., 1989) and therefore the RanGTP/RanGDP ratio is high within the nucleus. This gradient

of RanGDP/RanGTP across the nuclear envelope is used to drive protein trafficking back and forth through the NPC.

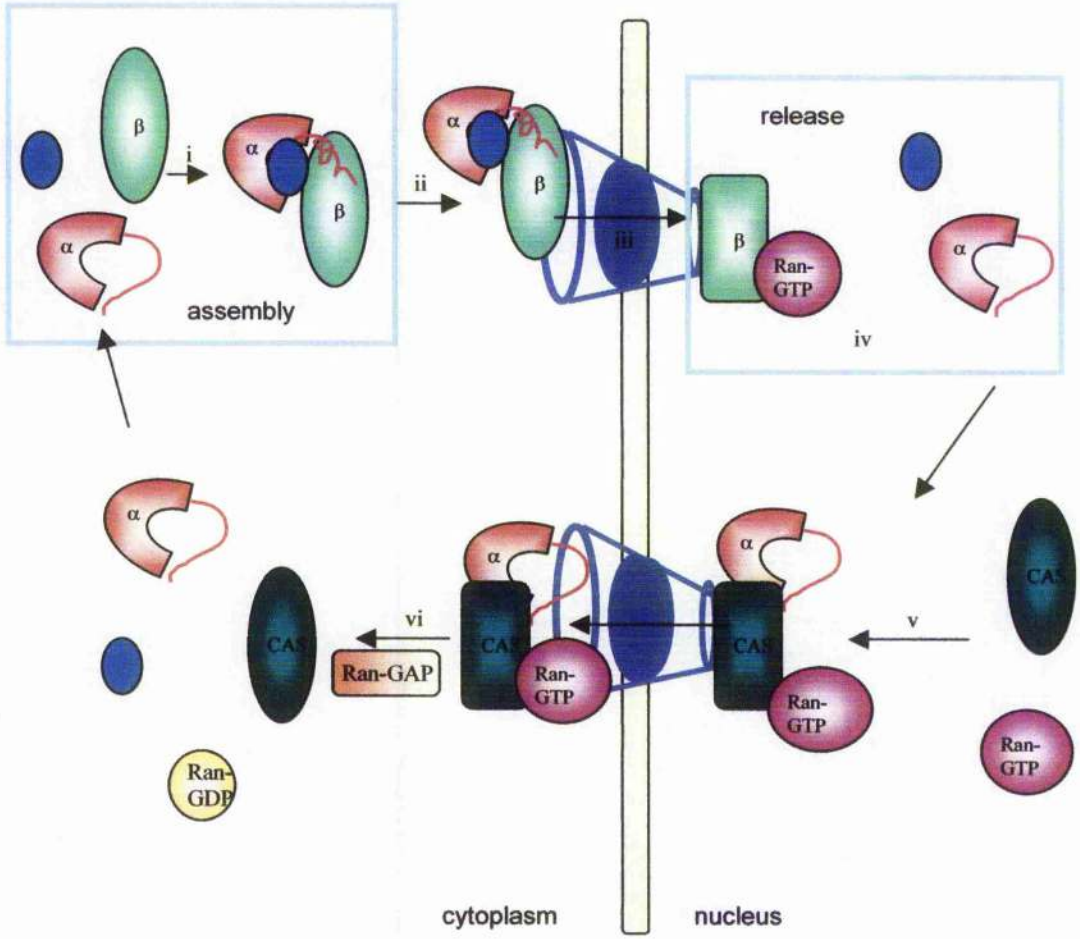
It is generally believed that importin-β is able to associate with either RanGDP or RanGTP and although it is possible that Ran hydrolysis could occur anywhere within the cell, it is tightly regulated by several proteins in close proximity to the NPC. On the cytoplasmic face of the NPC there is a 358kDa multi-domain protein, RanBP2 that acts as a scaffold where import and export complexes can assemble and disassemble. Importin-β and RanGAP1 are two of the several proteins known to bind to RanBP2 (Matunis et al., 1998). The currently accepted model for nuclear import, (as reviewed in Gasiorowski and Dean, 2003) is that the importin-α/β-RanGDP-NLS-cargo complex binds to RanBP2 on the cytoplasmic face of the NPC. The entire complex is then imported involving interactions with several NUPs. However the precise mechanism is not fully understood. Once within the nucleus, the high concentration of RCC1 exchanges the GDP with GTP causing the importin-α and cargo molecules to dissociate leaving the importin-β/RanGTP complex to translocate back through the NPC to the cytoplasm. A model of translocation is shown in Figure 1.6.

1.5.3 Nuclear Export

Nuclear export is also controlled by the RanGDP/GTP gradient along with a subfamily of importin-β, called exportins. The first identified exportin was CRM1, which has been found to recognise a leucine-rich nuclear export sequence (NES) (Fornerod et al., 1997). Proteins that contain a nuclear export sequence (NES) range from transcription factors to viral proteins (Gorlich and Kutay, 1999; Mosammaparast and Pemberton, 2004). In contrast to the process observed with nuclear import, RanGTP appears to promote the interaction between CRM1 and NES-containing cargo proteins (Chen and Greene, 2003; Gasiorowski and Dean, 2003). For example, IκB has a NES that is recognised by CRM1. Nuclear IκB binds to activated NFκB, the IκB/NFκB

Figure 1.6 Nuclear translocation of cNLS via Importin α/β complex

(i) Importin α (α) forms a ternary complex with importin β (β) and cargo (blue circles). **(ii)** The ternary complex docks at the nuclear-pore complex (NPC) and **(iii)** translocates into the nucleus. **(iv)** Binding of Ran–GTP triggers the dissociation of the ternary complex. **(v)** Importin α binds to the exportin CAS–Ran–GTP complex and is exported to the cytoplasm. **(vi)** Ran–GAP-stimulated hydrolysis of GTP by Ran triggers the dissociation of the exportin complex and releases free importin α into the cytoplasm for another transport cycle. The recycling of importin β to the cytoplasm, and Ran–GDP to the nucleus and its conversion to Ran–GTP are not shown.



complex then interacts with CRM1 and RanGTP enabling its subsequent trafficking through the NPC back into the cytoplasm. It has been shown that leptomycin B (LMB), which directly binds to CRM1 preventing its interaction with the NES (Fornerod et al., 1997; Fukuda et al., 1997; Kudo et al., 1998), inhibits the nuclear export of the IκB/NFκB complex (Huang et al., 2000).

1.6 Role of G-protein Coupled Receptors (GPCRs) in Inflammation

G-protein coupled receptors (GPCRs) constitute the largest family of integral membrane proteins involved in signal transmission and as such play a key role in immune and cellular responses. They can bind a diverse range of important inflammatory ligands including, chemokines, seretonin (5HT) and adenosine, making them an important target for potential anti-inflammatory therapeutics.

GPCRs are integral membrane proteins that consist of a single polypeptide chain containing seven transmembrane (TM) spanning hydrophobic α-helical regions with a hydrophilic extracellular N-terminus and an intracellular C-terminus which can vary in length. Despite the conservation of this original structure, sequence homologies between distinct GPCRs are generally restricted to the transmembrane domains of closely related receptor subtypes. Generally the ligand recognition site involves the extracellular domains of the receptor (both the N-terminus and the extracellular loops between the α-helices) and the pocket formed by the assembly of the seven TM helices, whereas interaction between the receptor and intracellular signalling partners (G-proteins) involves the intracellular loops and C-terminal tail (Gether, 2000; Wess, 1997; Wess, 1998). The GPCR superfamily can be further divided into different classes characterised by their ligand binding domains. The largest is Group A (rhodopsin-type receptors) and contains receptors for prostaglandins, prostacyclins and adenosine, along with the cell surface receptors for some viruses. Group B (secretin/glucagons receptors) contains receptors for distinct hormones and peptides (eg calcitonin, glucagon and anti-diuretic hormone).

Group C (metabotropic glutamate receptors) was described most recently and includes receptors for glutamate and the Ca^{2+} ion (Wess, 1998).

1.6.1 General GPCR Signalling

GPCR activation occurs following the binding of its ligand. This results in the signal transduction via a conformational change within the protein structure responsible for coupling to and activation of the associated G-protein. It is this associated G-protein that activates the downstream effector molecules to initiate intracellular signalling.

The structure of GPCRs has been elucidated from numerous mutagenesis studies involving rhodopsin and β_2-adrenergic receptors (β_2AdAR) and the crystal stucture of rhodopsin (Palczewski et al., 2000). It has been shown that the TM domains of GPCRs are more closely packed at the intracellular surface than at the extracellular surface enabling ligand binding and that the N- and C-terminals vary in length. In most receptors, the N-terminal domain contains the sequence Asn-X-Ser/Ther where X is any amino acid except proline (Hubbard and Ivatt, 1981) which are sites for possible N-linked glycosylation. Although it is not known if all possible sites are functional, it has been reported that some are necessary for receptor expression and ligand binding activity (Liu et al., 1993; Segaloff and Ascoli, 1993; Wess, 1998). The majority of GPCRs contain Cys residues in the first and second intracellular loops that are responsible for maintaining the tertiary structure of the protein via the formation of disulfide bonds as shown in rhodopsin and β_2AdAR (Dixon et al., 1987; Karnik et al., 1988). The C-terminal tail and the third intracellular loop typically contain numerous Ser and Thr residues that are potential phosphorylation sites for regulation by kinases such as GPCR kinases (GRKs) and/or second messenger kinases such as protein kinase A (PKA) and protein kinase C (PKC) (Dohlman et al., 1987; Kobilka, 1992). These residues are particularly significant for desensitisation of receptor signalling (Section 1.6.3.1). In addition, the third TM domain cytosolic region contains the highly conserved sequence Asp-Arg-Tyr

(DRY). This sequence, in conjunction with the second intracellular loop, is thought to be involved in receptor-G-protein coupling.

1.6.2 Receptor and G-protein Interactions

A lot is known about the structural features of GPCRs involved in ligand recognition and G-protein binding. However, the mechanisms underlying ligand-induced activation and subsequent G-protein coupling are still relatively unclear. The DRY motif is a highly conserved triplet of amino acids located at the cytosolic region of the third TM helix and the second intracellular loop (Probst et al., 1992). The importance of the DRY sequence has been described for several GPCRs including the M1 muscarinic receptor, α_{1B}-adrenergic receptor (α_{1B}-AdR), angiotensin IA receptor ($AT_{1A}R$) and the CXCR8 receptors (Damaj et al., 1996; Jones et al., 1995; Ohyama et al., 1992) The Arg residue within the DRY motif is fully conserved amongst all class 1 GPCRs as mutagenesis experiments with both the M1 muscarinic and α_{1B}-adrenergic (α_{1B}-AdR) receptors resulted in impaired signal transduction properties (Jones et al., 1995; Zhu and Toews, 1994). Interestingly, functional studies with the M1 muscarinic receptors have shown that a charge-conserving Arg for Lys mutation resulted in only a modest impairment of receptor function (Jones et al., 1995) which suggest that the conserved Arg residue presumably interacts with an anionic site on the G-protein.

1.6.3 Regulation of GPCR Function

The precise control of receptor expression and function is extremely important in the role that GPCRs play in inflammation. It is essential that they can accurately respond to rapid changes in agonist concentration. This is ensured by phosphorylation of specific Ser, Thr or Tyr residues by multiple protein kinases. The result is multiple protein-protein interactions that lead to receptor desensitisation, sequestration from the cell surface, recycling of the receptor back to the surface and in some cases down-regulation of the receptor.

1.6.3.1 Desensitisation

Desensitisation has been defined as the process whereby a GPCR-initiated response reaches a plateau and then decreases despite the continued presence of agonist (Freedman and Lefkowitz, 1996; Krupnick and Benovic, 1998). It can be further sub-divided into homologous and heterologous desensitisation. Homologous desensitisation causes the desensitisation of only agonist-occupied receptors, while in contrast; heterologous desensitisation describes the desensitisation of inactive and/or un-related GPCRs. Heterologous desensitisation is mediated by phosphorylation of specific residues by second messenger-activated kinases such as PKA and PKC, resulting in a decrease in the ability of the receptor to stimulate the associated G-protein (Benovic et al., 1985; Pitcher et al., 1992). In 1987, studies using the β_2AdR resulted in the purification of a kinase from bovine brain responsible for homologous desensitisation (Benovic et al., 1987). This kinase was termed β-adrenergic receptor kinase (βARK) but was later renamed as G-protein coupled receptor kinase 2 (GRK2) (Benovic, 1991). Subsequently, there have been seven mammalian GRKs cloned to date, termed GRK1-7. GRKs 1 and 7 are solely expressed in the eye whilst GRK4 is predominantly expressed within the testis (Penn et al., 2000; Pitcher et al., 1998). The other GRKs are ubiquitously expressed suggesting an important role for these kinases in mediating homologous desensitisation for a wide variety of GPCRs.

1.6.3.2 Sequestration

Sequestration is the process whereby agonist stimulated GPCRs, having been phosphorylated by GRKs, are removed from the cell surface. Typically this requires prior receptor phosphorylation and subsequent binding of an arrestin protein (Diviani et al., 1996; Ferguson et al., 2002; Pippig et al., 1993). Of the four known mammalian arrestins (arrestin1-4) that share 45% overall identity, arrestins 1 and 4 are found exclusively in the eye (Murakami et al., 1993; Shinohara et al., 1992). Arrestins 2 and 3 (Lohse et al., 1990; Sterne-Marr et al., 1993) are ubiquitously expressed and therefore play a general role in

sequestration of many GPCRs. The binding of arrestin to phosphorylated GPCRs can also result in the uncoupling of activated receptors from the G-proteins, thereby "arresting" (switching off) GPCR intracellular signalling (Bunemann and Hosey, 1999).

1.6.3.3 Down-regulation

Prolonged agonist exposure over a period of hours results in receptor down-regulation, defined as an overall decrease in total receptor number (Gagnon et al., 1998). It has been suggested from studies on the Thrombin receptor (Hein et al., 1994), the thyrotropin-releasing hormone receptor (TRHR; Petrou et al., 1997) and the cholecystokinin receptor type A (CCK_AR; Tarasova et al., 1997) that sequestered receptors traffic in an agonist-dependent manner from early endosomes to lysosomes where they undergo degradation.

After receptors and their ligands have been sequestered from the cell surface by clatherin-coated vesicles, the vesicles are uncoated and the internalised molecules are delivered to early sorting endosomes (Ferguson, 2001). Due to the acidic pH in early endosomes, the receptors and ligands dissociate and the receptors are either recycled back to the membrane for another round of agonist activation or degraded by the lysosome (Koenig and Edwardson, 1997). The effective regulation of receptor surface expression maintains the sensitivity of GPCRs to extracellular concentrations of agonist which is important in the activation of signal transduction pathways via G-proteins.

1.6.4 Constitutive Receptor Signalling

The concept of constitutively active GPCRs was first introduced by Costa and Hertz in 1989 and is now widely accepted within receptor pharmacology (Reviewed in de Ligt et. al., 2000, Costa and Herz, 2005). Constitutive activity of GPCRs is described as receptor signalling without agonist intervention. This exhibition of spontaneous receptor activity has lead to the observation that various ligands, previously considered as antagonists with no intrinsic activity,

actually can inhibit this spontaneous activity, appearing to possess 'negative' intrinsic activity. This phenomenon has been termed inverse agonism and the corresponding ligands are referred to as inverse agonists (Milligan G 2003, de Light et al., 2000). An important consideration during the classification of ligands based on their (negative) intrinsic activity, is that due to the large influence of receptor systems and experimental conditions (whole cells versus membranes, stoichiometry of receptors / G-protein, signalling proteins etc.) the same ligand may behave as an inverse agonist, a neutral antagonist or even a (partial) agonist (Greasley and Clapham, 2006).

Although virtually all GPCRs can be modified to display constitutive activity (Chalmers and Behan, 2002), many GPCRs display rather low levels of constitutive activity when expressed in recombinant systems and display greatly increased activity in the presence of agonist. For example, the mutation of Ala 293 on the α_{1b} adrenoreceptor to any other amino acid increased constitutive activity (Kjelsberg et al., 1992) which indicates a strong evolutionary pressure to maintain the wide-type form of the receptor in a nearly silent state.

There have been documented differences in the extent of constitutive activity among GPCRs, even between sub-types of the same receptor family (Milligan G., 2003). The question is whether these subtle distinctions are a natural way of fulfilling a specific role in signalling.

1.7 G-proteins

Although recent studies have revealed the existence of G-protein–independent signalling through certain GPCRs (Bockaert and Pin, 1999; Hall et al., 1999; Heuss et al., 1999; Hur and Kim, 2002), the common biochemical feature of these receptors is their interaction with G-proteins and the activation of downstream signalling cascades through a well documented and conserved molecular mechanism. The binding of agonist alters the conformation of critical domains of the TM helix pocket, which in turn causes changes in the

conformation of intracellular domains of the receptor. These changes promote the specific association of the receptor with a variety of heterotrimeric G-proteins. These are composed of an α-subunit interacting with a βγ-complex. Activation of the receptor promotes the exchange of a molecule of GDP by a molecule of GTP within the active site of the α-subunit. The binding of GTP causes the dissociation of the heterotrimeric complex, and both the GTP-bound α-subunit and the released βγ-complex are then able to interact with intracellular or membrane effectors of various signalling pathways. The intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP, restoring its initial inactive conformation along with its affinity for the βγ-complex (Susa, 1999). For many receptors, the localisation of the molecular determinants of the receptor involved in the coupling and activation of G-proteins has been investigated. These studies have highlighted the role of membrane-proximal regions of the second and third intracellular loops and of the C-terminus of the receptor in driving coupling. However, no consensus linear sequence for G-protein interaction has yet been clearly identified (reviewed in Gether, 2000; Wess, 1997; Wess, 1998).

The complexity and specificity of GPCR signalling partly relies on the existence of numerous closely related molecular species of the G-protein subunits (Downes and Gautam, 1999; Morris and Malbon, 1999). Numerous α-subunits have been identified from various genes and are classified into four families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12}$) according to its function (Hermans, 2003).

1.7.1 $G\alpha_s$

The $G\alpha_s$ family of G-proteins is defined by its ability to stimulate adenylyl cyclase activity leading to the generation of intracellular cAMP. $G\alpha_s$ and $G\alpha_{olf}$, the two members of this family, share 88% homology (Jones and Reed, 1989) and are substrates for ADP-ribosylation of Arg 201 found within the GTP-binding site (Kassis et al., 1982). This post-translational modification inhibits the intrinsic GTPase activity of the G-proteins, leading to the constitutive

activation of the initiation of cellular events that characterise the pathophysiology of cholera (Jones and Reed, 1989).

1.7.2 $G\alpha_i$

The $G\alpha_i$ family proteins comprise, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, the retinal $G\alpha_t$, $G\alpha_t$, two forms of the brain-specific $G\alpha$ subunit $G\alpha_{o1}$ and $G\alpha_{o2}$, along with $G\alpha_z$. Defined by the inhibition of adenylyl cyclase activity they can also target other downstream signalling components including certain sodium, potassium and chloride channels and PLCβ, (Moriarty et al., 1990). With the exception of $G\alpha_z$, all other members of this class contain a conserved C-terminal Cys residue that is the site of ADP-ribosylation catalysed by a toxin from *Bortadella pertussis*. This causes irreversible, uncoupling of the G-protein from its activating receptor leaving the subunit in a permanently inactive GDP-bound state, thus preventing further GDP/GTP exchange by active GPCRs.

1.7.3 $G\alpha_q$

The $G\alpha_q$ family contains five family members, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{16}$, and $G\alpha_q$. These closely related proteins are not substrates for either cholera toxin- or pertussis toxin-catalysed ADP-ribosylation. The $G\alpha_q$ subunits are notable regulators of the β-class of phosphoinositide-specific phospholipase C (PLC) PLCβ (Rhee, 2001). $G\alpha_q$ and $G\alpha_{11}$ are widely expressed in mammalian tissues, whilst in contrast the other members of the family are restricted to stromal and epithelial cells and are thought to activate specific members of the $PLC\beta_2$ family that also have a similar restricted pattern of expression (Morris and Malbon, 1999).

1.7.4 $G\alpha_{12}$

This family comprises of two 44kDa proteins $G\alpha_{12}$ and $G\alpha_{13}$, both of which are cholera toxin and pertussis toxin resistant. Ubiquitously expressed, they couple GPCR activation to mutiple downstream targets including the RhoGEFs which

activate Rho, a family of small GTP-binding proteins, and non-receptor tyrosine kinases such as Ras GTP-ase activating protein (Offermanns, 2003). They have also been reported to interact with cadherins, a class of adhesion molecules, causing the release of transcriptional activator β-catenin (Meigs et al., 2002; Meigs et al., 2001).

1.7.5 Gβγ Subunits

It is widely accepted that the Gβγ subunits can also target effector molecules that are involved in downstream receptor signalling (Wess, 1998). There have been 6 β-subunits and at least 12 γ-subunits reported (Clapham and Neer, 1997). The different β-subunits share a high degree of sequence homology whereas the γ-subunits are more structurally diverse. Gβγ-subunits are extremely stable and can only be separated by denaturisation and as such are regarded as one complex *in vivo*. Although theoretically the numbers of β- and γ-subunits could give rise to 72 different combinations, the reason for this diversity is as yet unclear as the majority of Gβγ subunits have been shown to exert similar functional properties (Clapham and Neer, 1997, Wess, 1998). It has also been reported that there is a βγ-binding site on GRK2 which allows for the regulation of GRK2 related receptor phosphorylation by promoting its translocation to the site of receptor-G-protein interaction and also inhibits Gβγ signalling (Lodowski et al., 2003).

1.8 Adenosine and Inflammation

Extracellular adenosine is an ubiquitous critical regulator of homeostasis and plays a central role in the maintenance of the cardiovascular and central nervous systems. It is released by Na^+-dependent transporters and is formed in the extracellular space by the breakdown of ATP during times of stress such as hypoxia (Klinger et al., 2002). Due to deamination and/or cellular uptake, adenosine has a limited half-life therefore hypoxia-induced increases in concentration only affect local adenosine receptor signalling (Klinger et al.,

2002). In hypoxic tissues, adenosine accumulates to high levels sometimes reaching concentration levels of 1164±415 pmol/min/g, approximately 20 times greater than basal levels (59±28 pmol/min/g) (Decking et al., 1997), triggering signalling cascades which enable the tissues to cope with both short- and long-term effects of oxygen and nutrient deprivation (e.g. stimulating endothelial cell growth and angiogenesis).

There is a plethora of data linking adenosine to inflammation. Adenosine has been shown to inhibit phagocytosis, the generation of toxic oxygen species, and adhesion to endothelial surfaces (Cronstein, 1994). A study by Morabito et al. (1998) identified the anti-inflammatory effects of methotrexate, used in the treatment of rheumatoid arthritis, and sulfasalazine to include an adenosine-dependent mechanism. Both these therapeutic drugs act via different mechanisms to increase the extracellular concentration of adenosine. Methotrexate inhibits the enzyme aminoimidazolecarboxamino-adenosineribonucleotide (AICAR) transformylase (Allegra et al., 1985; Baggott et al., 1986). The resulting accumulation of AICAR and its metabolites has a direct effect on at least two key enzymes, adenosine deaminase and AMP deaminase, with the end result of increased concentrations of adenosine and adenine nucleotides (Chan and Cronstein, 2002). The anti-inflammatory agent, sulfasalazine has been shown to promote adenosine release both in vivo and in vitro and that adenosine, acting at its receptors, mediates the anti-inflammatory effects of sulfasalazine in the murine air-pouch model of inflammation (Gadangi et al., 1996). Further reports have shown sulfasalazine inhibition of leukocyte accumulation does not have an effect on either prostaglandin synthesis or translocation of NFκB subunit p50 into the nucleus (Cronstein et al., 1999).

Various knockout mice studies, utilising knockout mice in either adenosine deaminase or the A_{2A} adenosine receptor, have shown that increasing available adenosine produced an exaggerated inflammatory response when compared with wild-type (Blackburn et al., 2000; Hershfield, 2005; Ohta and Sitkovsky,

2001). This is probably due, in part, to the expression of adenosine receptors on the majority of cells throughout the immune system (Thiel et al., 2003).

1.8.1 Adenosine Receptors

Adenosine receptors (ARs) belong to the Class 1A GPCR family and are known as purinergic receptors as they bind the purine adenosine. There are two main types of purinergic receptors: P_1 or adenosine receptors, and P_2 receptors. P_2 receptors recognise and bind nucleotides such as ADP, ATP, UDP and UTP and can be sub divided into two classes. The P_{2x} receptors are ligand-gated ion channels (Benham and Tsien, 1987), while the P_{2y} receptors are GPCRs (Abbracchio and Burnstock, 1994; Fredholm and Altiok, 1994). The P_1/ARs can be divided into four distinct subtypes, the A_1AR, A_{2A}AR, A_{2B}AR and A_3AR (Tucker and Linden, 1993).

All four of the ARs are GPCRs and as such consist of an extracellular amino-terminal domain linked to a cytoplasmic-terminal domain by seven transmembrane-spanning α-helices (Ji et al., 1998). The α-helices 2, 3 and 5 show a large degree of sequence identity and residues within these regions are reported as being crucial for ligand binding and specificity. A_1AR, A_{2A}AR and A_{2B}ARs contain two histine residues within the TM regions which are have been identified via mutations to leucine to be important in ligand binding (Jacobson et al., 1992; Klotz et al., 1998; Olah et al., 1992).

The ARs are N-linked glycoproteins and all bar A_{2A}AR, have Cys sites for palmitoylation within the carboxyl terminus (Linden, 2001). Glycosylation has no effect on the affinity of ligands for receptors but is believed to be involved in targeting freshly synthesised receptors to the plasma membrane (Linden, 2001). Multiple potential phosphorylation sites have been identified within the C-terminal tail of A_{2A}AR, A_{2B}AR and A_3AR while the A_1AR contains fewer phosphorylation sites as outlined below.

1.8.2 AR subtypes

1.8.2.1 A_1AR

The A_1AR is highly expressed within most areas of the brain, heart, liver and testies (Fredholm et al., 2001) and has been shown to signal through the $G\alpha_{i/o}$ G-protein pathways therefore inhibiting adenylyl cyclase activity. Activation of this AR has also been shown to mobilise intracellular Ca^{2+} following the activation of phospholipase C (Hansen et al., 2003) and activate PKCμ in a MEK1 and PI3-K dependent manner (Hill et al., 2003). Once activated the A_1AR desensitises slowly over periods of several hours to days due to a lack of GRK phosphorylation sites within its C-terminal tail (Palmer et al., 1996). The A_1AR has also been reported to constitutively inhibit adenylyl cyclase when over-expressed in Chinese Hamster Ovary (CHO) cells (Shryock et al.,1998).

1.8.2.2 $A_{2A}AR$

The $A_{2A}AR$ is highly expressed within most cells of the immune system, platelets, heart, lung and endothelium (Fredholm et al., 2001; Thiel et al., 2003). Classically, the $A_{2A}AR$ signals via the G_s family of G proteins leading to an activation of adenylyl cyclase and the generation of cAMP (Daly et al., 1983). However, it has been shown that there may be cell type-specific patterns of $A_{2A}AR$-activated signalling. In endothelial cells, the $A_{2A}AR$ activates the ERK pathway in a $p21^{ras}$-, rap1-, and MAP Kinase Kinase (MEK1)- dependent manner (Seidel et al., 1999; Sexl et al., 1997).

A unique feature of the $A_{2A}AR$ is its long cytoplasmic C-terminal domain, 122 amino acid residues in humans, guinea pig and dogs and 125 in rats, compared to the $A_{2B}AR$ which only consists of 36 amino acid residues. 12 out of the 122 amino acids are serine and theronine residues and in cell experiments have been shown to be rapidly phosphorylated following activation of PKC. However, PKC is thought to activate intermediary kinases rather than act directly on the receptor due to the findings that mutation of three consensus

PKC phosphorylation sites to alanine failed to inhibit PKC-stimulated phosphorylation (Palmer and Stiles, 1999). In the same study it was also reported that phosphorylation of threonine 298 on the canine A_{2A}AR is associated with the rapid desensitisation in response to agonist and multiple isoforms of PKC activated the downstream kinase responsible for the phorbitol ester PMA-mediated phosphorylation of A_{2A}AR (Palmer and Stiles, 1999). The C-terminal tail is also proline rich with 11 residues in two clusters at positions –10 from the C-terminus and between positions 341-357, which is indicative of possible interactions with SH3 domains (SH- src homology). It has been suggested that it is involved in regulating the coupling properties of the receptor or may represent an anchor for a modulatory component (Kinlay et al., 2001). A recent study has provided a functional link between the A_{2A}AR and the actin cytoskeleton (Burgueno et al., 2003). Direct interaction between α-actinin and A_{2A}AR was observed suggesting that agonist-mediated clustering and internalisation of the A_{2A}AR regulated by its C-terminus is dependent on an intact α-actinin/actin network.

The A_{2A}AR has been shown to be constitutively active in both whole cell experiments and *in vivo* (Maenhaut et al., 1990, Lendent et al., 1992, Klinger et al., 2002). Transgenic mice carrying a thyroglobulin-A_{2A}AR hybrid gene have been shown to efficiently express the A_{2A}AR within the thyroid which correlated with increased levels of cAMP in the thyroid of transgenic animals indicating that the A_{2A}AR acts *in vivo* as a constitutive activator of adenylyl cyclase (Lendent et al., 1992). In correlation with these results, studies on the C-terminal tail of the A_{2A}AR, in human endothelial cells and HEK293 cells, have shown that truncation of the C-terminus blunted the capacity of the A_{2A}AR to elevate cAMP in the absence of agonist (Klinger et al., 2002). The constitutive elevation of cAMP was only detectable within intact cells and the difference between C-terminal-truncated and full-length A_{2A}AR was lost in membranes, suggesting a cellular component binds the C-terminus regulating the efficiency of signal transfer in intact cells.

1.8.2.3 $A_{2B}AR$

The $A_{2B}AR$ is expressed in the caecum, colon, lung and blood vessels (Fredholm et al., 2001). Coupling to $G\alpha_s$ and $G\alpha_q$, the $A_{2B}AR$ can not only increase intracellular cAMP but can also initiate Ca^{2+} mobilisation and MEK/Ras-mediated ERK activation (Gao et al., 1999). The $A_{2B}AR$ on vascular endothelial cells is also thought to contribute to the nitric oxide-dependent component of vasodilation mediated by Ca^{2+}-dependent NO synthase activation (Linden, 2001). Although wild-type A_3AR is not constitutively active, mutants conferring constitutive activity have been identified and utilised to identify ZM241385, DPCPX and MRS1706 as inverse agonists (Beukers MW et al., 2004, Li, Q et al., 2007)

1.8.2.4 A_3AR

The A_3AR has been cloned from various species incuding rat, dog, sheep, rabbit and human (Fredholm et al., 2001; Salvatore et al., 2000) The A_3AR displays the greatest degree of difference between species, with the rat and human sharing only 72% sequence identity. This is reflected by the marked differences in pharmacology and local of expression. In rodents, the A_3AR is highly expressed on mast cells and in the testis with some expression in the brain (Fredholm et al., 2001). In contrast, the A_3AR expression in humans and sheep is limited to eosinophils. It also couples to $G\alpha_i$ and appears to have the same affinity to adenosine as the A_1AR. Activated A_3ARs are susceptible to phosphorylation by GRK family of kinases (types 2, 3 and 5), resulting in rapid desensitisation (Palmer and Stiles, 2000). Constitutively active mutants (CAMs) of the human A_3AR have been constructed which appear to favour adenylyl cyclase signalling rather than the PLC signalling pathway (Chen et al., 2001).

1.9 Evidence of a Role of Adenosine Receptors in Inflammation

It is widely accepted that inflammatory tissue damage is accompanied by the accumulation of extracellular adenosine in inflamed areas due to its release from non-immune and immune cells (Berne and Rubio, 1974; Bockman et al., 1975; Cronstein et al., 1985; Sitkovsky, 2003; Sullivan and Parker, 1976). Also contributing to the accumulation of adenosine is the release of rapidly metabolised adenosine diphosphate (ADP) and adenosine triphosphate (ATP) from various cells including platelets and endothelial cells (Linden, 2001). All of the AR subtypes, each of which play distinct roles in inflammation, can be activated by adenosine generated extracellularly, particularly on endothelial cells. It has been shown that the last step in the extracellular breakdown of ATP to adenosine is controlled by the cell membrane-anchored surface enzyme CD73/ecto-5'-nucleotidase, predominantly associated with the vascular endothelium, which catalyses the extracellular converstion of 5'AMP to adenosine (Zernecke et al., 2006). The genetic deletion of CD73 has recently been associated with an impaired generation of adenosine, demonstrating its importance in modulating vascular tone and in limiting inflammatory responses by attenuating leukocyte adhesion (Koszalka et al., 2004; Thompson et al., 2004; Zernecke et al., 2006). Several studies suggest that the four ARs have both pro-and anti-inflammatory properties dependent on the model system studied.

1.9.1 Inflammatory Effects of A_1AR Activation

The A_1AR has been shown to have both pro-and anti-inflammatory effects. A_1AR activation is reported to reduce hypersensitivity to heat and paw pressure in rodent models of inflammation and nerve injury (Li et al., 2003) and treatment with an A_1AR agonist improves the symptoms of arthritis in rat models (Boyle et al., 2002). A protective effect of the A_1AR has been suggested in studies of renal ischaemic-reperfusion injury in A_1AR knockout mice as these mice exhibit increased renal injury when compared to their wild type littermates (Lee et al.,

2004). In contrast, animal models of asthma it is suggested that the A_1AR is involved in mediating bronchospasm by a neutral mechanism which is independent of mast cell granulation (Meade et al., 2001). These data suggest that the different roles of the A_1AR observed within each animal model are mediated by alternative signalling pathways.

1.9.2 Inflammatory Effects of A_{2B}AR Activation

Little is known about the cell-specific expression of the A_{2B}AR *in vivo* or about the functional significance of the A_{2B}AR, the latter is due to a lack of specific agonists for this receptor. Despite these limitations, cell culture studies have indicated that A_{2B}AR is expressed in immune cells, endothelial cells and aortic vascular smooth muscle (Yaar et al., 2005). The A_{2B}AR has been implicated in several important biological events, including mediating vasodilation (Balwierczak et al., 1991; Martin, 1992), inhibiting growth of rat aortic smooth muscle cells (Dubey et al., 1996; Dubey et al., 2000) and increasing the production of cytokines, such as IL-6, by vascular cells (Feoktistov et al., 2002; Zhong et al., 2004). It has also been reported that the A_{2B}AR is involved in the pathogenesis of Alzheimers disease as A_{2B}AR expression was found to be reduced in rodent models of Alzheimers disease with induced neuro-inflammation which was prevented by treatment with a NO-releasing non-steroidal anti-inflammatory drug (Rosi et al., 2003). Pro-inflammatory effects are also reported in different models of inflammation. Activation of the A_{2B}AR by adenosine has been shown to increase the secretion of the pro-inflammatory cytokine IL-6 in *in vitro* studies of intestinal inflammation (Sitaraman et al., 2001). However the direct influence of A_{2B}AR on cytokine levels, inflammation and/or vascular adhesion has not yet been examined *in vivo*.

1.9.3 Inflammatory Effects of A_3AR Activation

The A_3AR has been shown to exhibit pro- and anti-inflammatory effects. *In vivo* studies of A_3AR knockout mice have indicated that activation of the A_3AR is able to inhibit LPS-induced TNFα production (Salvatore et al., 2000). This is

supported by reports that treatment with an A_3AR agonist was shown to decrease inflammatory cell infiltration into the colon and reduce cytokine and chemokine levels in mice models of colitis (Mabley et al., 2003). In contrast, A_3AR activation in basophils and mast cells is able to stimulate degranulation and the resulting bronchospasm that leads to asthma (Nagano et al., 2000; Tilley et al., 2003). It has also been reported that the A_3AR has a pro-inflammatory role as inflammation was greatly reduced in A_3AR knockout mice when compared to their wild type littermates (Wu et al., 2002). However due to the species-specific differences in the pharmacology of the A_3AR between rodents and humans, it is unclear whether these data can be relied upon to reflect the signalling responses in humans.

1.9.4 Anti-inflammatory Effects of $A_{2A}AR$ Activation

In contrast to the other ARs, multiple *in vivo* and *in vitro* studies show a potent anti-inflammatory effect of the $A_{2A}AR$. It was reported in 1989 that $A_{2A}AR$ activation could inhibit superoxide release from guinea pig and human eosinophils stimulated with opsonised zyosan (Yukawa et al., 1989). Further studies by Salmon and Cronstein, (1990), reported that the $A_{2A}AR$ selective agonist NECA could inhibit Fcγ R-mediated phagocytosis and superoxide generation in polymorphonuclear leukocytes (PMN) whereas the AR agonist 8-*p*-sulphenyltheophylline blocked this effect. It has also been reported that a decrease in the adhesion of fMLP-induced PMN to endothelial cells was detected using concentrations of adenosine and NECA sufficient to inhibit the generation of reactive oxygen species (ROS) (Cronstein et al., 1992). Studies in neutophils have indicated that this reduction was due to the inhibition of $\alpha_M\beta_2$ integrin expression (Wollner et al., 1993).

As mentioned earlier, the anti-inflammatory effect of the folate antagonist methotrexate is also adenosine dependent (Chan and Cronstein, 2002). It has been shown that injection of low concentrations of methotrexate can reduce leukocyte accumulation in carrageen-inflamed air pouches. This was found to

be due to the accumulated presence of adenosine following up-regulation of the adenosine transporter, in splenocytes (Cronstein, 1994). During the same study it was also found that inhibition of adenosine kinase could reduce leukocyte accumulation, an effect that was completely reversed by co-injection of adenosine deaminase.

The development of A_{2A}AR gene-deficient mice has provided the most valuable tool in studying the anti-inflammatory effects of the A_{2A}AR and have established the critical role of these receptors in several models of immune-mediated tissue damage *in vivo* (Ledent et al., 1997; Ohta and Sitkovsky, 2001). The absence of A_{2A}ARs on immune cells means that the accumulation of extracellular adenosine in inflamed areas is not able to trigger the production of intracellular cAMP, allowing the uninhibited and uniterupted activities of activated immune cells and for continued tissue damage (Sitkovsky et al., 2004). It has also been reported that the A_{2A}AR has a role in wound healing as mice lacking the A_{2A}AR form less dense granular tissue and fewer blood vessels during wound repair and surprisingly accumulate fewer leukocytes in response to inflammatory stimuli (Montesinos et al., 2002).

Due to these findings, the development of selective A_{2A}AR agonist is of great interest and many groups have successfully synthesised such compounds and demonstrated their anti-inflammatory potential (Lappas et al., 2005). Most A_{2A}AR selective agonists are 2-substitutions of the non-selective AR agonist NECA and include CGS21680 (Hutchison et al., 1989), HENECA (Cristalli et al., 1992), ATL-146e (Sullivan et al., 2001) and MRE-0470 (Glover et al., 2001). The selective activation of the A_{2A}AR has been shown to have significant protective effects in several models of inflammation causing a decrease in neutrophil transmigration into the cerebrospinal fluid in patients with endotoxin stimulated meningitis and attenuate the inflammation induced increase in the blood-brain barrier permeability (Sullivan et al., 1999), inhibition of oxygen radical production (Thiel et al., 2003) and inhibition of cytokine production such

as TNFα (Majumdar and Aggarwal, 2003). In addition, ATL-146e reduces joint destruction caused by septic arthosis and CGS 21680 can regulate HIV-1 transactivation regulating protein (Tat)-induced inflammatory responses (Cohen et al., 2004; Fotheringham et al., 2004).

The consistent anti-inflammatory role for A_{2A}AR signalling in a variety of different models of inflammation indicates a possible common mechanism of action. Various studies have shown that A_{2A}AR activation can inhibit the expression of pro-inflammatory cytokines (Bouma et al., 1996; Majumdar and Aggarwal, 2003) suggesting that this inhibition may occur at the level of transcription via a decreased accumulation of nuclear NFκB (Sands et al., 2004). The aim of this project was to determine where in the NFκB signalling pathway the A_{2A}AR was exerting its effects and also compare the effects of A_{2A}AR on certain NFκB regulated proteins involved in inflammation. The dissections of the pathways involved could lead to more specific drug design that may benefit a wide range of pro-inflammatory diseases.

Chapter 2
Materials and Methods

2.1 Chemicals and Suppliers

All reagents were of the highest grade commercially available and obtained from the following suppliers:

Abcam Ltd., Cambridge
Rabbit Anti-CREB (cat # ab3419), Rabbit Anti-Sp1 (cat # ab13370-50), Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (cat # ab8243)

Amersham Biosciences AB, SE-751 84, Uppsala, Sweden.
Glutathione-Sepharose beads™ 4B

Alexis Biochemicals, San Diego, CA92121/USA
Rabbit Anti-COX-2 (cat #210-712-1), Mouse Anti-COX-1 (cat #804-030-C500)

Assay Designs, 800 Technology Drive, Ann Arbour, MI 48108/USA
Correlate-EIA™ 6-keto-prostaglandin $F_{1\alpha}$ Enzyme Immunoassay Kit (cat # 900-004), Correlate-EIA™ prostaglandin E_2 Enzyme Immunoassay Kit (cat # 900-001)

BioRad Laboratories Ltd, Maylands Avenue, Hemel Hemstead
Protein assay dye reagent concentrate

BDH Laboratory Supplies, Poole BH15 1TD
Ammonium persulphate (APS), acetic acid, isopropylalcohol, methanol, potassium chloride, potassium hydroxide, sodium chloride

Cambrex BioScience Wokingham Ltd., BioWhittaker House, Workinghom, Berkshire
Endothelial Basal Media-2 ™, Singlequots™ (foetal bovine serum (FBS), hydrocortisone, fibroblast growth factor-B (hFGF-B), vascular endothelial growth

factor (VEGF), insulin-like growth factor-1 (IGF-1), ascorbic acid, epidermal growth factor (hEGF), gentamicin sulphate and amphotericin-B (GA-1000) and heparin), human umbilical vein endothelial cells (HUVECs), SeaPlaque ® agarose, phosphate-free Dulbecco's modified Eagle's medium (DMEM), cell culture grade phosphate-buffered saline (PBS)

Calbiochem-Novabiochem (UK) Ltd., Nottingham

Forskolin, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), pyrrolidinedithiocarbamate (PDTC), bisindolylmalemide 1 (BIM), emitine dihydrochloride, rolipram, 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U0126)

Cell Signalling Technology, Beverley, MA, U.S.A.

Mouse anti phospho-IκB-α (Ser 32/36) (cat # 9246), rabbit anti-MEK 1/2 (cat # 9122)

Duchefa Biochemie, Haarlem, Netherlands

Yeast extract, tryptone, microagar

Finnzymes Oy, Keilaranta 16 A, 02150 Espoo Finland

DyNAmo™ SYBR® green qPCR kit

Fisher Scientific, Loughborough, Leicestershire

4-2-hydroxyethl-1-piperazineethanesulphonic acid (HEPES), sodium dodecyl sulphate (SDS), ethylenediaminotetra-acetic acid (EDTA), dimethyl sulphoxide (DMSO), glacial acetic acid, ethidium bromide solution, glycine, ethanol, concentrated hydrochloric acid, sodium hydroxide, Tris (hydroxymethyl)-aminomethane (TRIS) base, sodium carbonate, sodium hydrogen carbonate, sodium di-hydrogen ortho-phosphate, di-sodium hydrogen ortho-phosphate, boric acid, chloroform, sucrose

GIBCO BRL Life Technologies, Paisley

Lipofectamine, OptiMEM

Inverclyde Biologicals, Strathclyde Business Park, Bellshill, Lanarkshire, UK

Protran nitrocellulose Schleicher and Schuell membrane (0.2μm pore size)

Melford laboratories, Chelsworth, Ipswich, Suffolk, UK

Dithiothreitol (DTT), isopropyl-β-D-thiogalactopyranoside (IPTG)

Molecular Probes, Invitrogen Ltd, Inchinnan Business Park, Paisley, UK

Alexa Fluor® 594 goat anti rabbit IgG (cat # A-11037)

New England Biolabs Inc., Beverley, MA U.S.A

Pre-stained protein molecular weight markers (ranging from 6.5-175kD), restriction enzymes PmeI, PacI

Novagen, Merck Biosciences LTD. Nottingham NG9 2JR, UK

Gene Juice

Perkin-Elmer Life and Analytical Sciences, Via Tiepolo, Monza, Italy

Enhanced chemiluminescence (ECL) reagents

Pierce, Rockford, IL, U.S.A.

Western Blot Stripping Solution, Slide-A-Lyzer ® Dialysis Cassette (0.5-3ml capacity)

Promega, Southhampton UK

Promega ™ Wizard Plus SV DNA mini-prep kit, AMV reverse transcriptase, ribonuclease inhibitor, oligo dT_{15} primer, Taq DNA polymerase, T4 Ligase

Qiagen, Crawley, West Sussex.

DNA plasmid maxi-prep kit

R&D systems, INC.

Sheep anti-human VCAM-1 (cat # AF809)

Reidel-de Haen, Seezle, Germany

Glycerol, calcium chloride

Roche Diagnostics GmbH, 68298 Mannheim, Germany

DIG Gel Shift Kit, 2nd Generation, Nylon membranes - positively charged

Santa Cruz Biotechnology, California, USA

Rabbit anti-IκB-α (S-21; cat # sc371, Sheep anti-Cdc42 (cat # sc6083), NFκB consensus oligonucleotide (cat # sc2505), mouse anti-Lyn (H-6) (cat # sc7274), rabbit anti-IκBε (cat # sc7155), rabbit anti-NFκB p65 (cat # sc109)

Serotec, Kindlington, Oxford, UK

Mouse anti human CD62E/CD62P (E-selectin) (cat # MCA883)

Sigma-Aldrich Company Ltd., Poole, Dorset

Leptomycin B, recombinant human interferon-γ (IFNγ), human tumour necrosis factor-α (TNFα), *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) Triton X-100, Tween-20, soybean trypsin inhibitor, benzamidine, bovine serum albumin (BSA), 30% (w/v) acrylamide / 0.8% (w/v) bis-acrylamide, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated anti-sheep IgG, horseradish peroxidase (HRP)-conjugated anti-goat IgG, thimerosal, bromophenol blue, agarose, ampicillin, paraformaldhyde, bichinchonic acid di-sodium salt (BCA), N,N,N`,N`-tetramethylethylenediamine (TEMED), phenlymethylsulphonyl fluoride (PMSF), foetal bovine serum (FBS), cell culture

grade trypsin, endothelial grade trypsin, (0.5g/L), penicillin/streptomycin, L-Glutamine, 3,3`5,5`-tetramethylbenzidine (TMB), sodium fluoride, sodium periodate, sodium potassium tartrate, deoxycholic acid (sodium salt), poly-D-lysine, Trizol Reagent, lysosyme, sodium orthovanadate, NP-40, diethyl pyrocarbonate (DEPC), phenol:chloroform:isoamyl alcohol (25:24:1;v/v/v), ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), brilliant blue G

Stratagene, Gebouw California, 1101 CB Amsterdam, The Netherlands
BJ5183-AD-1 electroporation-competent bacterial cells

Thermo Electron GmbH, D89077 Ulm, Germany
Oligonucleotide primers (see Table 2.3.1 for details)

pGEX/importin α1, 3, 5 and 7 constructs (Fagerlund et al., 2005) were gifted from Dr Riku Fagerlund, Dept. of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland.

Arkalone P was kindly gifted from Dr Ian Salt, Division of Biochemistry and Molecular Biology, IBLS, University of Glasgow, UK.

Sheep anti-p50/p105 polyclonal antibody (Hay and Nicholson, 1993) was kindly gifted from Prof. RT Hay, Division of Gene Regulation and Expression, School of Life Sciences, University of Dundee, UK.

Dried skimmed milk powder was obtained from a variety of commercial sources.

2.2 Cell Culture and Transfection Methods

2.2.1 Cell Maintenance

All cell types were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

HUVECs were maintained in endothelial basal medium supplemented with 2% (v/v) foetal bovine serum, 0.04% (v/v) hydrocortisone, 0.4% (v/v) fibroblast growth factor-B (hFGF-B), 0.1% (v/v) vascular endothelial growth factor (VEGF), 0.1% (v/v) insulin-like growth factor-1 (IGF-1), 0.1% (v/v) ascorbic acid, 0.1% (v/v) epidermal growth factor (hEGF), 0.1% (v/v) gentamicin sulphate and amphotericin-B (GA-1000), and 0.1% (v/v) heparin. Cells were routinely sub-cultured only until passage 6, as HUVECs have been shown in cell culture conditions to become less responsive over time to cytokines thereby changing the regulation of expression of different cellular proteins such as adhesion molecules (Muller et al., 2002). When confluent, cells were washed in 2 x 20ml sterile PBS (phosphate-buffered saline) and then treated briefly with 2ml endothelial grade trypsin in order to detach the cells. The trypsin was then neutralised with 10ml of spent media and the contents transferred to a 50ml centrifuge tube. Cells were centrifuged for 5 min at 200 x g and the supernatant was discarded. The cell pellet was gently resuspended in medium at a dilution factor suitable to establish a cell density which could be reliably counted using a standard haemocytometer (typically 1:10). Wells were then seeded at an appropriate level according to the analysis performed as indicated in the figure legends. Typically a 6-well plate would be seeded with 4×10^5 cells /well and a 10cm dish would be seeded with 8×10^4 cells, ready for infection the following day. A minimum of 1×10^4 cells was used to maintain the cell line in a fresh $150cm^2$ tissue culture flask to which 11ml of fresh medium was added.

Human embryonic kidney 293 cells (HEK293) were maintained in DMEM supplemented with 10% (v/v) FBS, 1mM L-glutamine, 100units/ml penicillin and

100μg/ml streptomycin. Confluent monolayers were washed in 2 x 20ml sterile PBS and then treated with 1ml trypsin. Cells were then returned to the incubator for a few minutes before the flask was disrupted to dislodge the cells. Cells were then typically diluted 1:8, 7ml of which was used in experimental analysis and of which 1ml was used to maintain the cells to which 9ml of fresh medium was added.

2.3 Molecular Biology

2.3.1 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) was prepared, autoclaved and allowed to cool before the addition of ampicillin antibiotic at the final concentration of 50μg/ml. The liquid LB agar was then poured into 90mm-diameter Petri dishes, allowed to solidify, then left to sweat overnight at room temperature to rid the plates of excess moisture. Plates were then stored at 4°C for a maximum of 2 weeks until required.

2.3.2 Transformation of competent *E.coli* (XL1 Blue and BL21 strains)

Approximately 30-50ng of plasmid DNA was added to a plastic 13ml Falcon round-bottomed tube on ice. Once thawed 50μl /tube of competent *E.coli* was immediately added and the mix incubated on ice for 30min. The tubes were heat shocked at 45°C for 45sec then immediately returned to ice. 0.5ml of LB per tube was added and the tubes shaken at 37°C for 1 hour. 0.1ml of the transformation mix was then plated out onto LB-Amp plates (see section 2.3.1) and incubated overnight at 37°C.

2.3.3 Preparation of plasmid DNA

Transformed colonies picked from agar plates using sterile pipette tips, were used to inoculate 10ml of LB broth supplemented with 50μg/ml ampicillin, and grown in a shaking incubator at 37°C overnight. Plasmid DNA was then

isolated using the Promega Wizard plus SV miniprep purification system as per the manufacturer's instructions. For larger quantities of plasmid DNA, the initial 10ml culture was then used to inoculate a 500ml culture containing ampicillin and grown overnight. Plasmid DNA was isolated using the Qiagen Maxi Kit system as directed by the manufacturer's instructions. The concentration of double stranded DNA obtained was calculated based on the assumption that 1 absorbance unit (A_{260}) is equivalent to 50μg/ml of double stranded DNA. An absorbance ratio (A_{260}/ A_{280}) greater than or equal to 1 indicated good quality DNA that was free of contamination with protein.

2.3.4 Digestion of plasmid DNA

1-2μg of purified plasmid DNA was digested in a sterile microfuge tube containing the appropriate enzyme buffer and 2-4 units of enzyme as per the manufacturer's instructions. In certain cases it was necessary, due to incompatible buffer restrictions, to purify the linearised plasmid DNA from the first digestion, before digestion with the second enzyme and buffer. This was achieved by phenol extraction and ethanol precipitation. The volume of digested DNA was made up to 150μl with endonuclease free H_2O. In the fume hood, 50μl phenol:chloroform was added, the microfuge tubes were well shaken and incubated at room temperature for 5 min. The microfuge tubes were well shaken prior to centrifugation at 13,000g for 5 min at room temperature. The aqueous upper layer was carefully pipetted into a fresh microfuge tube and centrifuged for a further 5 min at 13,000g at room temperature. The supernatant was transferred to a fresh microfuge tube and 1/10 volume of 3M sodium acetate and 3 volumes of ice-cold absolute ethanol were added to the sample. Following 30 min incubation on ice, samples were centrifuged at 13,000g for 30 min at 4°C and the supernatant removed. The DNA pellet was washed once in 50μl of 70% (v/v) ethanol, centrifuged at 13,000g for 5 min at 4°C and air-dried. Restriction fragments were resolved on a 1% (w/v) agarose gel containing 2.5μg/ml ethidium bromide run at 75mV for 20-30 min in TAE buffer pH 8.0 (40mM Tris-acetate, 1mM EDTA, and 0.1% (v/v) glacial acetic

acid). Samples of the restriction fragments were resolved on a 1% (w/v) agarose gel containing 2.5μg/ml ethidium bromide run at 75 mV for 20-30 min in TAE buffer (40mM Tris-acetate, 1mM EDTA, and 0.1% (v/v) glacial acetic acid).

2.3.5 Preparation of RNA

10cm dishes were seeded with HUVECs at a density of 8 x 10^5 cells/dish and were treated the next day as indicated in the figure legends. Following treatment, the dishes were placed on ice and the cell monolayer washed twice with ice-cold PBS after which all PBS was drained off. The cells were then harvested by re-suspension into 1ml Trizol and stored in a cryovial at -80°C overnight. Samples were defrosted at room temperature and briefly pulsed to prevent phenol splashes. 0.2ml of chloroform was added and the samples were shaken vigorously by hand for 15 seconds. Following an incubation at room temperature for 3min, the samples where then centrifuged at 12000g for 15min to separate the RNA, DNA and protein fractions. The upper aqueous phase was carefully pipetted into a fresh microfuge tube and the RNA precipitated by mixing with 0.5ml isopropylalcohol and incubation at room temperature for 10min. Samples were then centrifuged at 12000g, room temperature for 10min, the supernatant was discarded and the pellet was washed with 75% (v:v) ethanol and vortexed. Samples were then centrifuged at 12000g, room temperature for 5min. The supernatant was discarded and the pellet left to air-dry for 5-10min. Once dry the pellet was resuspended in 50μl RNAse free water and incubated for 10min at 60°C to ensure the RNA was completely dissolved. The concentration of RNA obtained was calculated based on the assumption that 1 absorbance unit (A_{260}) is equivalent to 40μg/ml of RNA. An absorbance ratio (A_{260}/ A_{280}) equal to 1.8 indicated RNA free from protein and DNA contamination. RNA was stored at -80°C until analysis.

2.3.6 Reverse transcription

First strand cDNA was synthesised from RNA (section 2.3.6) using AMV reverse transcriptase. 2μg RNA was made up to 15μl with DEPC-treated water

and 300ng of oligo dT_{15} primer. Samples were heated at 70°C for 3 minutes and then immediately placed on ice to cool. A mixture of 5x reverse transcriptase buffer (supplied with kit) and dNTPs was made and added to each reaction to give final concentrations of 50mM Tris-HCL (pH 8.3 @ 25°C), 40mM KCL, 8.75mM $MgCl_2$, 10mM DTT, 0.1mg.ml acetylated BSA and 1mM of each dNTP. 20U Ribonuclease inhibitor and 50U AMV reverse transcriptase were then added, the samples mixed by pipetting and incubated at 42°C for 3hrs. The cDNA generated was stored at -20°C until PCR analysis was performed.

2.3.7 qRT-PCR of prepared cDNA

Real-time PCR was carried out using DNA Engine Opticon 3™ system (MJ Research Inc.). Prior to this, standard PCR has been carried out to confirm that primers caused amplification of only one product of the correct size (human COX-2 amplicon size of 290bp and human GAPDH amplicon size of 150bp;). cDNA equilivant to 200ng total RNA was amplified using DyNAmo™ SYBR® green qPCR kit (Finnzymes). The kit provides a 2x master mix comprising modified Thermus brockianus DNA polymerase, SYBR Green 1 fluorescent dye-optimised PCR buffer, 5mM $MgCl_2$ and dNTP mix. Data were analysed using the Opticon analysis software.

For each run, a PCR master mix was prepared, which included primers for the gene of interest (final concentration of 1μM for both GAPDH and COX-2; Table 2.3.7.1) with the master mix from the DyNAmo ™ SYBR® green kit. This was pipetted into a 96-well plate (20μl per well) and appropriately diluted cDNA samples (5μl) were added to give a final volume of 25μl. All samples were run in quadruplicate and standards in duplicate. The Opticon was preheated to 95°C to minimise the risk of primer-dimer formation, and a denaturing step followed of 10min at 95°C. Each cycle consisted of the following: an amplification step of 95°C for 10 seconds, followed by 60°C for 20sec, an extension step of 72°C for 20 seconds, a step to ensure primer dimers were

melted at 72°C for 20 seconds, followed by a fluorescence reading. This was repeated a further 39 times, to give a total of 40 cycles. Melting curves were constructed by taking readings every 0.2°C from 65°C to 95°C. The cooling step consisted of the plate being held at 4°C until the end of the run.

2.3.7.1 COX-2 and GAPDH primer details

Target Protein	Sequence (5' – 3')	Primer position
COX-2 (forward)	GCC CAG CAC TTC ACG CAT CAG	659-679
COX-2 (reverse)	AGA CCA GGC ACC AGA CCA AAG ACC	925-948
GAPDH (forward)	GAA GGT GAA GTG CGG AGT C	1245-1260
GAPDH (reverse)	GAA GAT GGT GAT GGG ATT TC	1445-1464

To determine efficiency of the PCR reaction and to enable relative quantification of genes, a standard curve was performed. For each analysis, a standard curve was constructed using serial log dilutions of cDNA from within the experiment and the sequence of interest was plotted so that cycle number correlated with the amount of product formed after each cycle. The first standard was made from 5µl of cDNA from the sample that was expected to have the highest quality of the gene of interest present (e.g. HUVECs stimulated with 10ng/ml TNFα for 10hrs). The relative COX-2 mRNA expression was normalised to GAPDH mRNA expression, which was also quantified by real-time PCR as described for COX-2.

2.3.8 Propagation and purification of human A_{2A} adenosine receptor adenovirus

In order to express myc-tagged human A_{2A}AR in HUVECs, an adenoviral gene transfer approach was used. This is because it achieved much greater

expression when compared with commercially available transfection reagents, electroporation or calcium phosphate-mediated transfection methods (Teifel et al., 1997). The propagation and purification of the Ad-A_{2A}AR/GFP and Ad-GFP were carried out in accordance with the protocols of Nicklin et al. (2001) using the AdEasy system described by (He et al., 1998).

Lab stocks of the pAdTrackCMV/mycA_{2A}AR shuttle construct (Sands et al., 2004) was cut at the PmeI site within the vector which exposed the inverted terminal repeats, and co-transformed with the viral backbone construct pAdEasy1 into E.coli rec+ strain BJ5183 by electroporation (2kV, 25μF, 200Ω). Recombination-positive clones identified by growth on ampicillin plates were screened by digestion with PmeI yielding a large (~30kb) fragment and a smaller (3-4.5Kb) fragment. These colonies were then expanded and the cDNA isolated. The plasmid cDNA was then linearised with PacI and 4.5μg was transfected using 15μl of GeneJuice onto low-passage HEK293 cells in 25cm^2 tissue culture flasks. The presence of the gene encoding GFP in a separate open reading frame allows monitoring of viral expression by fluorescence microscopy. 6 days post infection, cells were removed from the flask and collected in a sterile centrifuge tube and pelleted by centrifugation at 200 x g for 5 min at room temperature. The pellet was resuspended in 1.5ml of cold sterile PBS and transferred to a cryovial. The cells were then disrupted by four freeze/thaw cycles in a dry ice/methanol bath. Cleared lysate from this was then used to infect two T-150 flasks of 50-60% confluent HEK293 cells, grown in DMEM medium. Virus particles from these cells were prepared three days later as before, and used to infect a larger scale culture of 20 x T-150 flasks of HEK293 cells. From this large scale preparation, virus was isolated as per the method of Nicklin and Baker (2001). Briefly, dislodged cells were pooled and pelleted by a brief centrifugation at 250g for 10 min at room temperature. The supernatant was then transferred to sterile vials for future infection. The pellet was resuspended in 1.5ml of sterile PBS and subjected to four freeze/thaw cycles in order to lyse the cells and release the virus particles. The preparation

was then added to a discontinuous caesium chloride gradient set up in a 13ml sterile ultracentrifuge tube, in order to separate intact virus particles from other cellular debris and empty viral capsids. Turbulence caused by friction under braking would disrupt the separated adenoviral layer so the samples were subject to centrifugation at 100,000g for 90min at 8°C with zero deceleration. The opaque adenovirus band was then isolated by syringe extraction and added into a Slide-A-Lyser that allowed efficient overnight dialysis at 4°C in 1 L of dialysis buffer (51.2μM Tris, 10μM EDTA, 15mM NaCl) which was changed three times. The dialysed adenovirus was then diluted in a 1:1 (v/v) ratio with sterile virus storage buffer (10mM Tris-HCl (pH8), 100mM sodium chloride, 0.1% (w/v) BSA, 10% (w/v) glycerol) and stored at -80°C in 40μl aliquots.

2.3.9 Preparation of GST-Importin α 3-immobilised Sepharose beads

10ml of LB Amp broth (LB broth supplemented with 50μg/ml ampicillin) was inoculated from a glycerol stock of BL21 *E.coli* transformed with pGEX importin α-3, which was then grown overnight, shaking at 37°C. This starter culture was then used to inoculate 400ml LBAmp, which was grown shaking at 200rpm for 5 hours (or until OD_{600}=0.3 or greater) at 21°C. The cells were then induced by addition of IPTG to a final concentration of 1mM, then grown shaking at 200rpm for 4 hours at 21°C. The bacteria were then harvested by centrifugation at 6700g for 15 minutes, the supernatant discarded, cells resuspended in 20ml Lysis Buffer (50mM HEPES, pH7.4, 150mM NaCl, 5mM EDTA and 1% (v/v) Triton-X-100) and incubated at room temperature for 30mins. The sample was then probe sonicated to ensure efficient cell lysis and then centrifuged at 27000g for 30 minutes to pellet insoluble material. The cleared lysate was then added to 0.6ml 50% (v/v) glutathione-Sepharose bead suspension and placed on a rotating wheel at 4°C for 1 hour to allow the fusion protein to bind. The beads were then washed three times with 10ml PBS, following the final wash, resuspended in 50% (v/v) glycerol in PBS supplemented with protease inhibitors (0.1mM PMSF, 10μg/μl soybean trypsin inhibitor and 10μg/μl

benzamidine) and stored at -20°C. The same procedure was utilised for the preparation of GST-immobilised Sepharose beads which was used as a control.

2.3.10 SDS protein assay for immobilised GST Fusion proteins on Sepharose Beads.

20µl of prepared bead suspension was pippeted into a microfuge tube, briefly centrifuged for 20 sec at 4°C and supernatant removed using a 1ml syringe. The protein was eluted from the beads by the addition of 20µl of 2% SDS sample buffer and incubated at 37°C for 30min. Sample was briefly centrifuged at room temperature and the protein containing supernatant was removed via a Hamilton syringe into a fresh microfuge tube. Standard concentrations of BSA ranging from 0.2-2µg and 5µl and 10µl of eluted protein was analysed by SDS-PAGE using a 10% (w/v) resolving gel. The gel was stained with Coomassie stain (3mM Coomassie Brilliant Blue G, 45% (v/v) methanol, 10% (v/v) acetic acid) for 1hr and washed overnight in destain (50% (v/v) methanol, 5% (v/v) acetic acid). The stained gel was then scanned and the density of the protein bands were used to determine a straight line from which the concentration of protein immobilised on GST-Sepharose beads could be determined.

2.4 Laboratory Techniques

2.4.1 Protein concentration determination using the bicinchoninic acid (BCA) protein assay.

Duplicate 0.01ml samples of known BSA standards in the range 0-2mg/ml and unkown protein samples were added to a 96-well plate. 0.1ml BCA solution (1% (w/v) 4,4 dicarboxy-2,2 biquinoline disodium salt, 2% (w/v) sodium hydroxide, 0.95% (w/v) sodium bicarbonate pH 11.25, 0.08% (w/v) copper (II) sulphate) was then added to each well. Protein concentration-specific reduction of Cu^{2+} to Cu^{1+} allows the bicinchoninic acid sodium salt to bind the Cu^{1+} ion forming an intense purple colour allowing measurement of the absorbance at 492nm. Colour was therefore allowed to develop at room temperature for 30min and the absorbance of the standards was used to determine a straight line from which unknown protein concentrations could be calculated (Smith et al., 1985).

2.4.2 Protein concentration determination using Bradford's reagent

Duplicate 0.01ml of known BSA standards in the range of 0-2mg/ml and unknown protein samples were added to a 96-well plate. 0.01ml of Bradford's reagent was then added to each well. Binding of protein to the dye component of the Bradford reagent, Brilliant Blue G, causes a shift in the absorption spectra of the dye from 465nm to 595nm. Colour was therefore allowed to develop for 10-20 min and the OD_{600} of the standards and samples was measured. The absorbance of the standards was used to determine a straight line from which unknown protein concentrations could be calculated.

2.4.3 Discontinuous SDS-PAGE and Immunoblotting

Samples prepared for SDS-PAGE were equalised for protein using the bicinchonic acid assay described in Section 2.4.1. Pre-strained protein markers (Invitrogen Rainbow Markers, range 6.5-175kDa) in sample buffer (50mM Tris (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001% (w/v) of bromophenol

blue) were prepared in the electrophoresis to determine protein molecular mass. 30µl of sample, equalised for protein and volume, was then subjected to discontinuous SDS-PAGE using a 6 cm 10% (w/v) polyacrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH 8.8), 0.1% SDS, 3% (v/v) glycerol, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and a 2cm 3% (w/v) stacking gel (3% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% SDS, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED). Electrophoresis was carried out using BioRad Mini-Protean III gel electrophoresis systems in running buffer (27.4mM Tris, 0.19M glycine, 0.1% (w/v) SDS) at 150 V until the bromophenol blue dye front reached the end of the gel. Proteins were then transferred electrophoretically onto a nitrocellulose membrane at 400mA for 45 min in transfer buffer (24.7 mM Tris, 0.19M glycine in 20% (v/v) methanol). The nitrocellulose membrane was then removed, trimmed and briefly washed in PBS before incubating for 1hr at room temperature in Blotto (5% (w/v) skimmed milk, 0.1% (v/v) Tween-20 in PBS) to block non-specific binding of antibody. The membrane was then briefly washed in PBS before being transferred to a plastic bag containing 2ml of the relevant primary antibody diluted in Blotto as indicated in the Figure Legends. This was incubated at 4°C overnight on a rotating platform. The membranes were then rinsed briefly in PBS before being washed three times in Blotto for 10 min. Membranes were then washed again briefly in PBS before being transferred to a bag containing 2ml of the appropriate HRP-conjugated secondary antibody in Blotto. This was then incubated for 1hr on a rotating platform at room temperature. The membrane was then washed three times for 10min in Blotto and then washed a further twice in PBS for 10min. Membranes were then exposed to an enhanced chemiluminescence procedure. HRP-specific oxidative degeneration of the substrate from the bound secondary antibody causes emission of light at 428nm, detected by Kodak X-OMAT blue X-ray film to visualise immunoreactive proteins. Occasionally a higher stringency Blotto was required to minimise non-specific reactivity and provide clear visualisation of specific immunoreactive

proteins. In this case Blotto/Triton (5% (w/v) skimmed milk, 0.2% (v/v) Triton X-100 in PBS) was used to incubate the primary antibody and subsequent washes and High Detergent Blotto (10% (v/v) Blotto 1.02% (v/v) Triton X-100 and 0.1% (w/v) SDS in PBS) was used to dilute the secondary antibody.

2.4.4 Determination of cell surface protein expression by enzyme-linked immunoabsorbant assay (ELISA).

2×10^4 HUVECs/well were seeded in a 96-well plate. The following day, cells were treated with the appropriate concentrations of cytokine/lipopolysaccharide (LPS) for the time indicated in the Figure Legends. The assay was stopped by transferring the plate to ice and cells were washed gently three times with 200µl PBS. Cells were then fixed with 0.1ml of 4% (w/v) paraformaldehyde in 5% sucrose (w/v) in PBS (pH 7.2), covered and incubated overnight at 4°C. The following day, cells were washed gently three times with 200µl PBS and then incubated for 1hr at room temperature in 0.1% (w/v) BSA in PBS in order to block non-specific antibody binding sites. Cells were then washed gently three times with 200µl PBS before incubation with appropriate primary antibody at 1:1000 dilution in PBS/0.1% (w/v) BSA for 2hr at room temperature. To assess non-specific binding, an antibody raised in the same species as the specific antibody was also used at 1:1000 dilution, (E-selectin – Lyn (H-6), VCAM-1 – Cdc42) in wells treated in parallel. Cells were then gently washed three times with 200µl PBS before the addition of the appropriate HRP-conjugated secondary antibody at 1:1000 dilution in PBS/0.1% (w/v) BSA for 1hr at room temperature. Cells were then gently washed five times with 200µl PBS, before the addition of 100µl 3,3`5,5`-tetramethylbenzidine (TMB). The colour was allowed to develop for 5-15min at room temperature and the A_{600} determined using a plate reader.

2.4.5 Separation of Cytosolic and Nuclear fractions from treated HUVECs

1×10^5 cells were seeded out into 10cm dishes and then treated as described in the Figure Legends. Following cell treatments, the dishes were placed on ice,

the cell monolayers were gently washed three times with ice-cold PBS, and scraped into 1ml PBS before transfer to a microfuge tube. The cell suspension was centrifuged at 14 x g for 4 min at 4°C and the supernatant removed. The pellet was resuspended in 400µl of Buffer A (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EDTA 1mM DTT, pH7.9 with KOH) and allowed to swell on ice for 15mins. 25µl of 10% (v/v) NP40 was then added and the mix vortexed. The microfuge tube was then pulse centrifuged in the micro centrifuge for 10sec and the cytosolic fraction (supernatant) carefully removed and stored in a chilled microfuge tube at -80°C. The pellet was washed four times with 400µl of Buffer A. Following the fourth wash, the pellet was resuspended in 50µl Buffer B (20mM HEPES, 450mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, pH7.9 with NaOH), vortexed and incubated with shaking on ice for 15 min. The microfuge tube was then centrifuged at 10,000g for 15min at 4°C. The nuclear fraction (supernatant) was then transferred to a sterile chilled micofuge tube and stored at -80°C.

2.4.6 Electromobility Shift Assay (EMSA)

Nuclear fractions of treated HUVECs (section 2.4.5) were assayed for protein concentration using a Bradford Assay (as section 2.4.2) as the DTT present in Buffer B interferes with the BCA assay.

A double-stranded DNA probe (20ng/µl) containing the NFκB consensus sequence GGG GAC TTT CCC was used to detect any induced NFκB transcription factors. This procedure was undertaken utilising DIG Gel Shift Kit, (2^{nd} Generation) as per manufacturer's instructions (Roche). In brief, 100ng of NFκB consensus sequence oligo was labelled, aliquoted and stored at -20°C. Nuclear fractions were equalised for protein content in a total volume of 5µl and bound to 8ng of the labelled probe. The samples were run on a non-denaturing 6% (w/v) PAGE gel in 0.5 X Tris-borate-EDTA (TBE) buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA, pH8.0), then transferred to a positively charged Nylon membrane, at 400mA for 45 minutes in 0.5 X TBE buffer. The membrane

was then fixed by irradiation with UV light, blocked with kit blocking solution, probed with 75mU/ml anti-digoxigenin- alkaline phosphatase (Fab fragments congujated to AP) and the bands visualised by chemiluminescence detection.

2.4.7 Incident-light Fluorescence Microscopy

2×10^5 cells were seeded onto coverslips and then treated as described in the Figure Legends. Following cell treatments, cell monolayers were washed three times in 1.5ml PBS and then fixed in 1.5ml of 4% (w/v) paraformaldehyde in 5% (w/v) sucrose/PBS for 10min. Cells were then washed in 1.5ml PBS and blocked in blocking solution (3% (w/v) skimmed milk, 0.15% (v/v) Triton X-100 in PBS). The primary anti-MEK1/2 antibody was used at a 1:100 dilution in blocking solution and the Alexa 594-conjugated anti-mouse IgG was used at 1:400 dilution in blocking solution. 100µl of antibody solution was then spotted onto Nescofilm and the coverslip placed cells down onto the antibody solution and incubated for 1hr at room temperature. Cells were then washed three times in PBS and then placed onto 100µl of Alexa 594-conjugated secondary antibody on Nescofilm and incubated for 1 hr at room temperature. The coverslips were then washed three times in PBS prior to being thoroughly air dried and mounted onto microscope slides with 40% (v/v) glycerol in PBS.

Cells were observed using a Zeiss Axiovert 40 CFL microscope with HBO 50 illuminator (Zeiss, Oberkochen, Germany) using a Zeiss Plan- x 20 lens. GFP was detected via 515-540nm band pass filter. Alexa-594 conjugated antibodies were excited at 542nm and detected with a long pass band filter at 590nm. The images were adjusted with Zeiss LSM software.

2.4.8 Determination of Prostaglandin concentration by enzyme-linked immunoabsorbant assay (ELISA).

Prostaglandin E_2 and prostacyclin concentration levels in spent media were determined by Correlate-EIA™ Enzyme Immunoassay Kit. Prostacyclin has a short half life of 60 minutes so it is typically monitored by measurement of 6-

keto-$PGF_{1\alpha}$ which is produced by non-enzymic hydration of prostacylin and has been shown to be stable (Ylikorkala and Makila, 1985). 2 x 10^5 HUVECs cells/well were seeded out into 12-well plates and treated as described in the Figure Legends. Following cell treatment, the plates were placed on ice and the spent media removed to microfuge tubes and stored at -80°C. Samples were defrosted on ice and all ELISA reagents allowed to equilibrate to room temperature. The ELISAs were carried out as per manufactur's instructions, using 100µl of samples in duplicate and the plate read at A_{405} on a plate reader. The background reading from the lysates and the buffers were subtracted from the standards and test sample readings. From these readings a standard curve was produced using GraphPad Prism, from which the concentrations of Prostaglandin E_2 or 6-keto-prostaglandin $F_{1\alpha}$ were determined. These concentrations were then normalised to the control TNFα reading which was set at 100% and analysed using GraphPad Prism.

2.4.9 GST Pulldown Assay to detect importin α3-p65 interaction

8 x 10^5 HUVECs were seeded out into 10cm dishes which were then treated the following day as described in the figure legends. Following cell treatment, the dishes were placed on ice, the spent media removed and the cells washed twice with 10ml ice-cold PBS. The dishes were drained and the cells harvested in 250µl Lysis Buffer (50mM HEPES, pH7.4, 150mM NaCl, 5mM EDTA and 1% Triton-X-100) supplemented with 100µM sodium orthovanadate and protease inhibitors (0.1mM PMSF, 10µg/µl soybean trypsin inhibitor and 10µg/µl benzamidine) and transferred to a microfuge tube. The samples were placed on a rotating wheel at 4°C for 1 hr to ensure total lysis of the cells and then centrifuged at 20000g for 15 min. The soluble supernatant was transferred to a fresh microfuge tube and a BCA assay carried out to determine the protein concentration of the samples. (see Section 2.4.1). The samples were equalised for protein concentration and ~200µl was added to 5µg of GST-importin α3 immobilised to glutathione-Sepharose beads and incubated on a

rotating wheel at 4°C for 2hrs. The GST complexes were isolated by brief centrifugation and the supernatant discarded. The beads were then washed three times with 1ml lysis buffer. Bound proteins were eluted from the beads by the addition of 40μl electrophoresis sample buffer and incubation at 65°C for 15 minutes. The eluate and lysates were analysed by SDS-PAGE using 10% (w/v) resolving gels, transferred to nitrocellulose membranes. Membranes were stained with ponceau strain to ensure equal levels of GST-fusion protein had been used during the pull-down. Membranes were then blocked with Blotto supplemented with 0.1% (v/v) Tween-20 (section 2.4.3). Importinα3-bound p65 was detected by immunoblotting with the appropriate primary antibody as described in the appropriate figure legends.

2.4.10 HUVEC membrane preparation and ^{125}I-ZM241385 saturation binding

All procedures were on ice unless stated otherwise. Confluent 10 cm dishes of HUVECs were washed three times with cold PBS prior to scraping into 5 ml/dish PBS. Following centrifugation at 200g for 2 min, the isolated cell pellet was resuspended in 1 ml lysis buffer (10 mM sodium Hepes, pH 7.5, 5 mM EDTA) and lysed by 20 up-and-down strokes of a glass Dounce homogeniser. The membrane pellet was isolated by centrifugation at 48,000g for 15 min and initially resuspended in 0.2 ml binding buffer (50 mM sodium Hepes, pH 6.8, 10 mM magnesium chloride). Following the removal of duplicate 10μl aliquots for protein determination, the membrane suspension was diluted to a final volume of 4 ml with binding buffer supplemented with 1 unit/ml adenosine deaminase prior to further homogenisation and immediate use in binding assays.

Binding studies were performed in duplicate in a 0.25 ml final volume containing 0.15 ml membrane suspension, 50 μl ^{125}I-ZM241385 (ranging from 0.25 to 4-8 nM final concentrations, prepared and purified by high performance liquid chromatography to a specific activity of 2200 Ci/mmol by Dr Tim Palmer as described in Palmer et al., (1995) and 50 μl of either distilled deionised water or NECA at a final concentration of 50 μM (to define non-specific binding).

Incubations were carried out with shaking for 1 hr at 37°C by which time equilibrium had been reached. Bound radioligand was isolated by rapid vacuum filtration over 0.3 % (v/v) polyethylimine-soaked glass fibre filters followed by three 3 ml washes with ice-cold binding buffer supplemented with 0.03 %(w/v) CHAPS detergent to minimise non-specific binding. Following quantitation of filter-bound radioactivity using a gamma-counter, saturation curves were generated by fitting data to a one site hyperbola using Graphpad Prism software and from which K_d and B_{max} values were derived.

2.4.11 Statistical Analysis

Statistical analysis was carried out using Student's t-test using GraphPad Instat 3 as indicated in the Figure Legends. Significance was assessed as $p<0.05$.

Chapter 3

Effect of $A_{2A}AR$ gene transfer on the induction of inflammatory markers in HUVECs

3.1 Introduction

There is an obvious requirement to control prolonged or inappropriate inflammatory responses which contribute to the pathogenesis of many pro-inflammatory diseases such as ischemia-reperfusion injury (Sullivan et al., 2000) and atherosclerosis (Greaves and Channon, 2002). Regulated interactions between endothelial cells and leukocytes are fundamental to the inflammatory response as they trigger further downstream events including cytokine, chemokine and growth factor release, surface expression of adhesion molecules and expression of other pro-inflammatory proteins such as COX-2 (Section 1.2).

COX-2 is an inducible pro-inflammatory marker that catalyses the synthesis of the prostaglandin endoperoxide, PGH_2. This is the rate determining step in the production of prostaglandins that regulates vascular tone and tissue protection (Wu et al., 2005). Induced by pro-inflammatory stimuli including TNFα, LPS and IFNγ (Tanabe and Tohnai, 2002), COX-2 over-expression is associated with several inflammatory diseases including atherosclerosis, rheumatoid arthritis and sepsis (Crofford et al., 2000; Turini and DuBois, 2002; Zhang et al., 1997). In these cases inflammation is not resolved, thereby creating a severe pro-inflammatory state. The standard treatment for patients with pro-inflammatory diseases used to be via the pain relievers known as nonsteroidal anti-inflammatory drugs (NSAIDs). However most NSAIDs are considered non-selective due to their action of blocking both COX enzymes, resulting in irritation of the upper gastrointestinal tract due to COX-1 inhibition. The result of this was that selective COX-2 inhibitors were developed and about five years ago were approved for treatment (Mitchell and Warner, 2006). However in September 2004, Merck withdrew its COX-2 inhibitor *Vioxx* from the market following reports that selective COX-2 inhibitors were associated with an increased risk of myocardial infarction in susceptible patients (Bombardier et al., 2000; Caughey et al., 2001; Mitchell and Warner, 2006). A study by

Caughey et al., (2001) reported that induction of COX-2 in the endothelium resulted in increased synthesis of PGI_2 but not TXA_2, favouring an anti-thrombotic state. This implies that up-regulation of COX-2 in the endothelium may represent an important protective mechanism against vascular injury or insult. Therefore, understanding the cellular mechanisms regulating mediators of leukocyte interaction and COX-2 induction would provide an insight into possible future drug targets.

It is well documented that the A_{2A} adenosine receptor (A_{2A}AR) has an anti-inflammatory effect not only in numerous pharmacological studies (Linden, 2001) but also through A_{2A}AR-knockout mice studies *in vivo* (Gomez and Sitkovsky, 2003; Ohta and Sitkovsky, 2001). Previous work undertaken in our lab has shown that A_{2A}AR-mediated inhibition of Nuclear Factor κB (NFκB) activation is a critical aspect of its anti-inflammatory signalling properties and that the molecular basis of this inhibition is cell type-specific (Sands et al., 2004), although the precise mechanism that underlies this important property is not yet fully understood. As discussed in the introduction, endothelial cells line the entire cardiovascular system and are involved in the pathology of many disease states that involve platelet activation or leukocyte infiltration into underlying tissue. Immortalised endothelial cell lines are commonly used to examine endothelial/leukocyte interactions and negate the variability associated with primary endothelial cells. It is widely accepted that a heterogeneity of endothelial function (Yang et al., 1991) and phenotype (Page et al., 1992) exists depending on the vascular bed of origin. One of the major documented differences between endothelial cells, as far as inflammatory processes are concerned, relates to responses to cytokines. Human umbilical vein endothelial cells (HUVECs) were used as a model of inflammation *in vitro* in our studies as they are relatively tractable compared to other human endothelial cell types whilst still able to respond similarly to ECs from other vascular beds to cytokine exposure (Lidington et al., 1999). However while tractable, it is difficult to introduce cDNA into primary endothelial cells which

could be problematic as poor transfection efficiencies could lead to a masking of any inhibitory effects of A_{2A}AR expression. With transfection efficiencies of as low as 2-10% commonly generated with standard transfection methods (Teifel et al., 1997), a replication-deficient recombinant adenovirus encoding a *myc*-His-tagged human A_{2A}AR was used to ensure consistently high levels of A_{2A}AR transfection (see Results).

We have previously demonstrated that in HUVECs, A_{2A}AR gene transfer suppresses NFκB activation in response to stimuli such as TNFα and LPS by reducing the nuclear accumulation of p50/p65 heterodimers (Sands et al., 2004). To fully assess any functional significance of this phenomenon, the effects of A_{2A}AR gene transfer in HUVECs on the accumulation of three important gene products whose induction is controlled at least in part by NFκB was compared. These are, A) COX-2, the rate limiting enzyme in the formation of prostaglandins in response to pro-inflammatory stimuli (Smith et al., 1996b; Wu, 1995), B) the type 1 glycoprotein E-selectin, which is involved in the rolling phase of leukocyte recruitment and C) the immunoglobulin-like adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), which binds firmly to $\alpha_4\beta_1$ integrin expressed on infiltrating leukocytes to arrest leukocyte on activated ECs prior to their translocation into the underlying tissue.

3.2 Results

Before investigating the effect of $A_{2A}AR$ gene transfer on the accumulation of NFκB-regulated pro-inflammatory proteins, the effect of various NFκB-activating pro-inflammatory stimuli on protein induction was examined. LPS and TNFα are known to activate NFκB via different cell surface receptors (see Sections 1.3.1 and 1.3.3). To compare the ability of each stimulus to induce COX-2, confluent monolayers of HUVECs were treated with LPS, TNFα and IFNγ either on its own or in combination for 24hrs. Cell lysates were then subjected to SDS-PAGE followed by immunoblotting with anti-COX-2 antibody as described in section 2.4.3. Figure 3.1 shows that significant COX-2 expression was induced solely by TNFα and that the presence of IFNγ did not alter TNFα-mediated expression. It is reported that the other cyclooxygenase isoform, COX-1 is constitutively expressed in HUVECs (Caughey et al., 2001). Thus COX-1 levels are stable regardless of any pro-inflammatory stimulation and can be used as an appropriate loading control. In summary, it is clearly shown that the greatest COX-2 induction is achieved in the presence of TNFα and therefore subsequent experiments were undertaken using this cytokine.

To identify the concentration of TNFα which provided the greatest induction, confluent monolayers of HUVECs were treated with increasing concentrations of TNFα from 10pg/ml to 10ng/ml for 24hrs. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-COX-2 antibody as described in section 2.4.3. It can be seen in Figure 3.2, that significant TNFα-mediated COX-2 induction was detectable at 0.1ng/ml TNFα increasing to a maximum response observed following treatment with 10ng/ml TNFα. As COX-2 induction did not saturate with the concentrations of TNFα used, it was not possible to calculate an accurate EC_{50} from these data. Thus for maximal TNFα-mediated COX-2 induction, a concentration of 10ng/ml was utilised in subsequent experiments.

Figure 3.1 Effect of various ligands on COX-2 induction

$4x10^5$ HUVECs/well were seeded into a 6 well plate. 24hrs after seeding, cells were stimulated with different pro-inflammatory stimuli (1μg/ml LPS, 10ng/ml TNFα, 10units/ml IFNγ, 1μg/ml LPS + 10units/ml IFNγ, 10ng/ml TNFα+ 10units/ml IFNγ) for 24hrs. Cells were harvested, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3

The graph show combined results from 3 experiments, where as the blot is representative of one experiment.

*** = P<0.001 when compared to vehicle

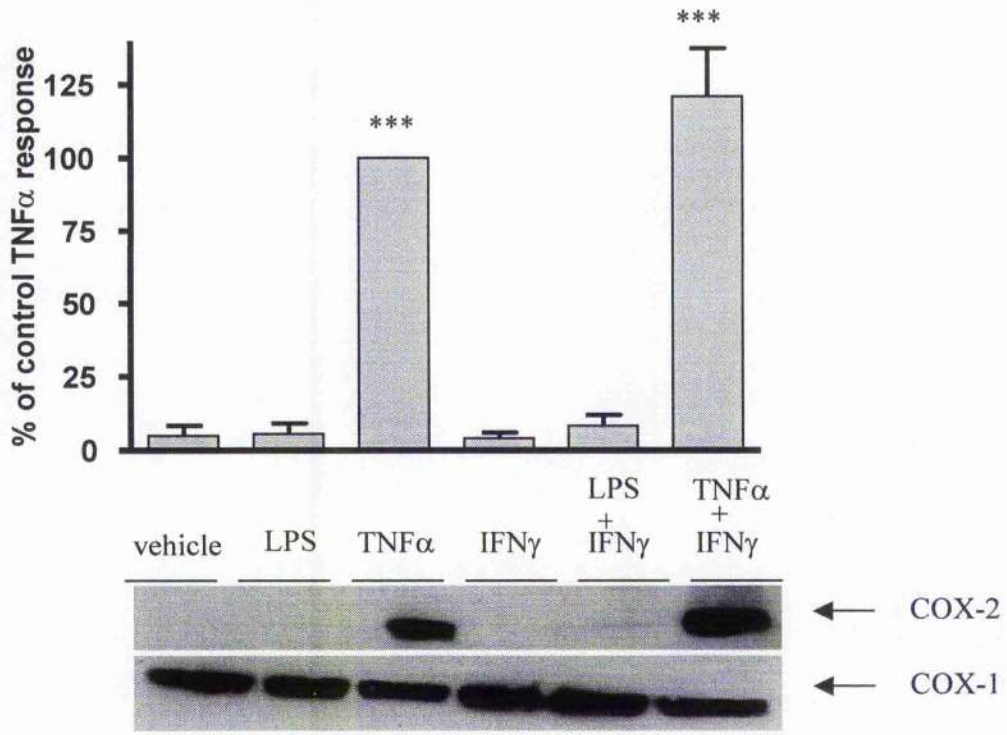


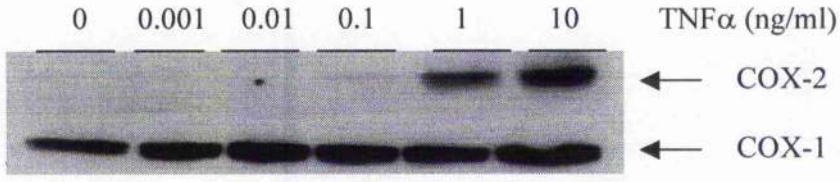
Figure 3.2 Effect of increasing concentrations of TNFα on COX-2 Induction

$4x10^5$ HUVECs / well were seeded into a 6 well plate. 24hrs after seeding, cells were stimulated with the indicated concentrations of TNFα for 24hrs. The cells were then harvested, equalised for protein concentrations and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3

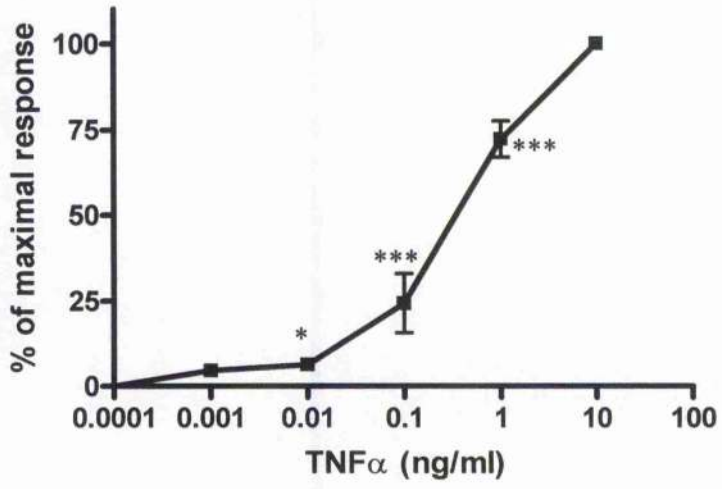
Panel A shows representative blots of one experiment whilst panel B shows the combined results from n=3 experiments.

Statistical significance observed * = P<0.5, *** = P<0.001 when compared to vehicle.

A



B



The effect of inhibitors and stimuli on any cell can differ at various time points studied. Thus, to determine which time point resulted in optimal induction, confluent monolayer of HUVECs were stimulated with 10ng/ml TNFα at various time-points over a 48hr period. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-COX-2 antibody. Figure 3.3 shows that a statistically significant increase in TNFα-mediated levels of COX-2 induction was detectable after 12hrs, reaching a maximum at 24hrs and although levels decreased after 48hrs they were still found to be significant.

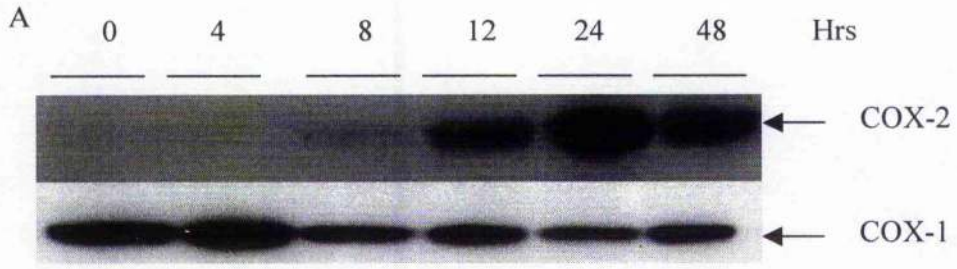
It has already been shown that the $A_{2A}AR$ decreases NFκB accumulation in the nucleus (Sands et al., 2004). The COX-2 promoter has been shown to contain several potential transcription regulatory elements, including an NF-IL6 motif, two NFκB sites and a CRE motif (Chun and Surh, 2004). To determine which signalling pathways were involved in COX-2 induction, confluent monolayers of HUVECs were pre-treated with the following signalling inhibitors for 30mins. SB203580, a p38 MAPK inhibitor (Lee et al., 1994), U0126, an inhibitor of MEK1 or 2, the upstream activators of ERK 1 or 2 (DeSilva et al., 1998), pyrrolidinedithiocarbamate (PDTC) an inhibitor of NFκB activation (Pan et al., 1995), N-acetyl-$_L$-cysteine (NacCys) as a control for the antioxidant properties of PDTC (Tsai et al., 1996) and bisindolylmalemide 1 (BIM) a PKC inhibitor (Kiss et al., 1995). Following pre-treatment, the cells were then stimulated with 10ng/ml TNFα for 24hrs and the cell lysates subjected to SDS-PAGE and immunoblotting with anti-COX-2 antibody. As shown in Figure 3.4, only pre-treatment with SB203580 resulted in a reduction of TNFα-mediated COX-2 induction suggesting that at 24hr COX-2 induction is solely regulated by the p38 MAPK pathway. This was unexpected as COX-2 is known to be regulated by NFκB (Deng et al., 2003). NFκB is a transcription factor, therefore it might be expected that regulation of transcription would occur earlier than 24hrs. Thus, to identify whether NFκB is involved at an earlier time point the experiment was repeated but stimulating with TNFα for only 8hrs (Figure 3.5). This showed that at this earlier time-point there is a statistically significant decrease in

Figure 3.3 Time-course of COX-2 induction in response to maximal concentration of TNFα

$4x10^5$ HUVECs/ well were seeded into a 6 well plate. 24hrs after seeding, cells were stimulated with 10ng/ml TNFα at the shown time points from 48hrs. Cells were then harvested, lysed and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3

Panel A shows representative blots of one experiment whilst panel B shows the combined results from n=3 experiments.

Statistical significance observed ** = P<0.01, *** = P<0.001 when compared to vehicle.



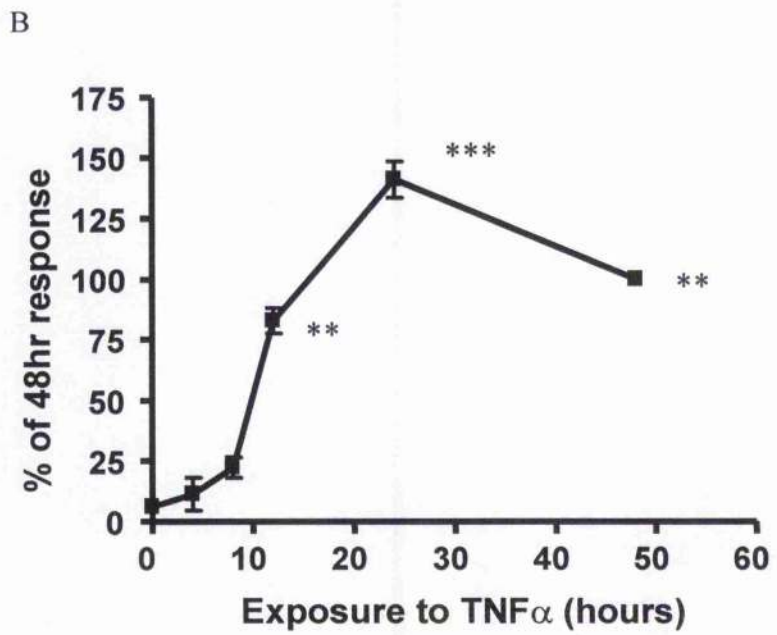


Figure 3.4 Effect of signalling inhibitors on TNFα induced COX-2 induction after 24 hrs

$4x10^5$ HUVECs / well were seeded into 2x 6 well plates, 24hrs after seeding, the cells were pre-treated with either vehicle, 10μM SB203580 (p38 MAPK inhibitor), 100μM U0126 (ERK inhibitor), 100μM PDTC (NFκB inhibitor), 100μM NAcCys (PDTC control), 5μM BIM (PKC inhibitor) for 30mins then stimulated ± 10ng/ml TNFα for 24hrs. Cells were harvested, lysed, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.

The graph shows the combined results from 3 experiments, whilst the blots are representative of one experiment.

*** = P<0.001 when compared to vehicle +TNFα

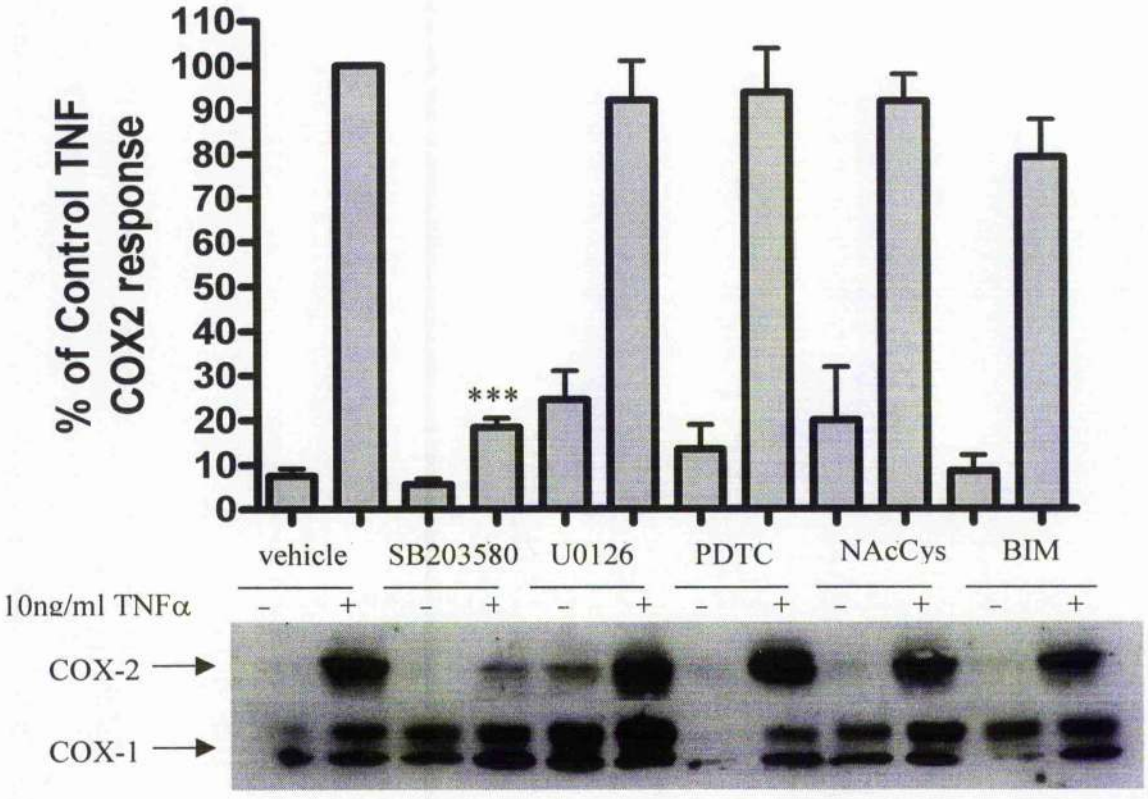
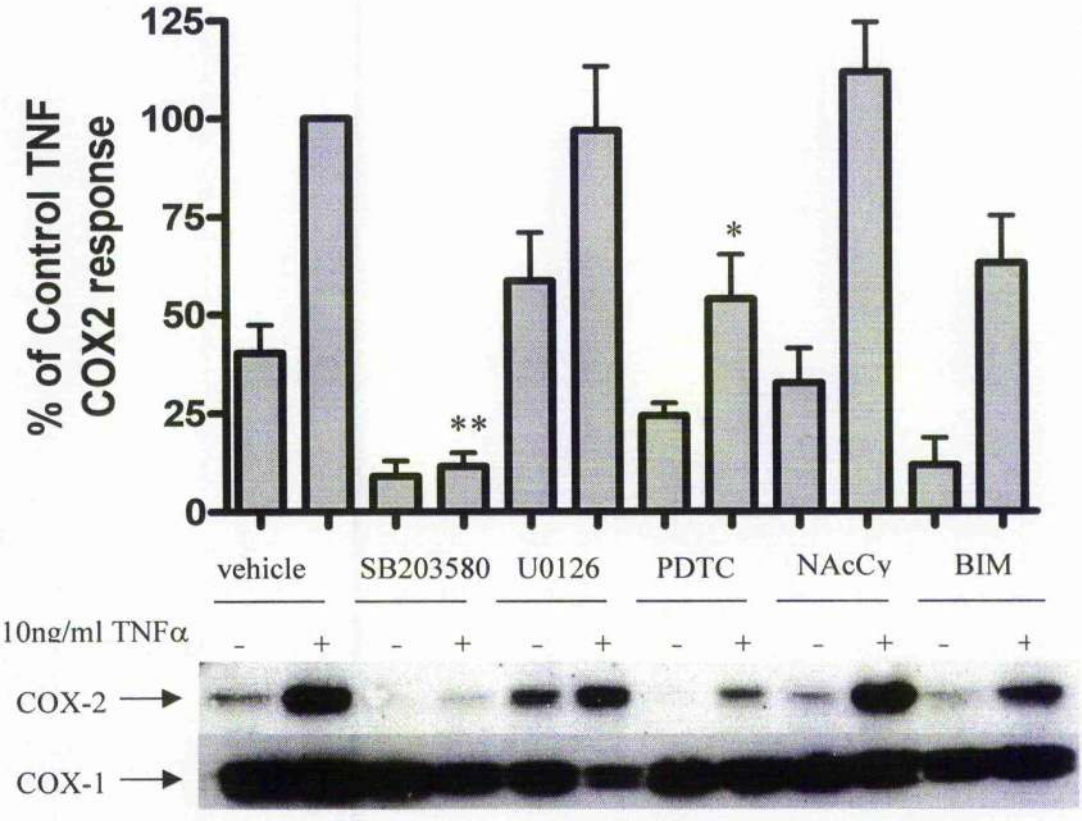


Figure 3.5 Effect of signalling inhibitors on TNFα induced COX-2 induction after 8 hrs

$4x10^5$ HUVECs / well were seeded into 2x 6 well plates, 24hrs after seeding, the cells were pre-treated with either vehicle, 10μM SB203580 (p38 MAPK inhibitor), 100μM U0126 (ERK inhibitor), 100μM PDTC (NFκB inhibitor), 100μM NAcCys (PDTC control), 5μM BIM (PKC inhibitor) for 30mins then stimulated ± 10ng/ml TNFα for 8hrs. Cells were harvested, lysed, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.

The graph shows the combined results from 3 experiments, whilst the blots are representative of one experiment.

** = P<0.01, * = P<0.05 when compared to vehicle + TNFα



COX-2 induction in the presence of PDTC, as well as in the presence of SB203580. The graph indicates that PKC could also be involved but this reduction proved to be statistically insignificant. Thus, TNFα-mediated COX-2 induction is regulated largely by two pathways; at 8hrs by NFκB and p38 MAPK pathways but only by the p38 MAPK pathway at later time-points.

Other pro-inflammatory markers regulated by NFκB are specific adhesion molecules required for leukocyte trafficking. To identify whether A_{2A}AR gene transfer exerts any significant effect on other κB regulated genes, two of these adhesion molecules, E-selectin and VCAM-1, were analysed. As E-selectin is a cell surface protein the effects of the various pro-inflammatory stimuli (LPS, TNFα and IFNγ) were examined via cell surface ELISA as described in section 2.4.4. It can be seen in Figure 3.6 that LPS stimulation caused a significant increase in E-selectin induction but TNFα stimulation caused an even greater increase. The presence of IFNγ either on its own or in the presence of either LPS or TNFα does not appear to have any significant effect on induction. Previous work undertaken by the lab had already characterised the optimum conditions of TNFα-mediated E-selectin induction (4hr stimulation with 10ng/ml TNFα; A. F. Martin, unpublished results).

Like COX-2, the E-selectin promoter consists of various transcription promoter elements including three -κB sites and a CRE/ATF-like domain (Collins et al., 1995). To determine whether any response observed was due to a κB effect, confluent monolayers of HUVECs were pre-treated for 30mins with the same panel of signalling inhibitors as in Figure 3.4-3.5. The cells were then stimulated with TNFα for 4hrs and subjected to ELISA with anti-E-selectin antibodies as described in 2.4.4. As shown in Figure 3.7, only treatment with the NFκB inhibitor PDTC produced any significant reduction in E-selectin induction. Therefore, TNFα-mediated E-selectin induction is solely NFκB-dependent. Like E-selectin, VCAM-1 is a cell surface adhesion molecule

Figure 3.6 Effect of different pro-inflammatory stimuli on E-Selectin induction

$4x10^4$ HUVECs/ well were seeded into a 96 well plate. 24hrs after seeding, cells were stimulated with different pro-inflammatory stimuli (1μg/ml LPS, 10ng/ml TNFα, 10units/ml IFNγ, 1μg/ml LPS + 10units/ml IFNγ, 10ng/ml TNFα+ 10units/ml IFNγ) for 4hrs. Cells were assessed for E-selectin induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

* = P<0.05, *** = P<0.001 when compared with vehicle

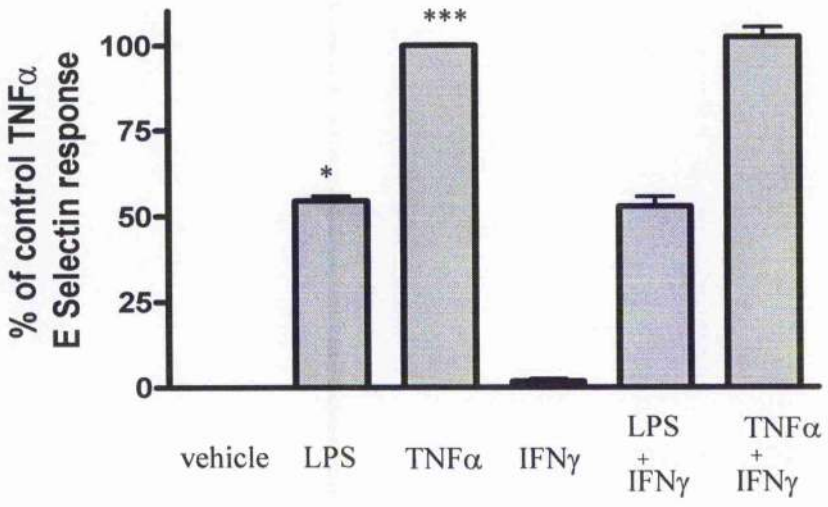
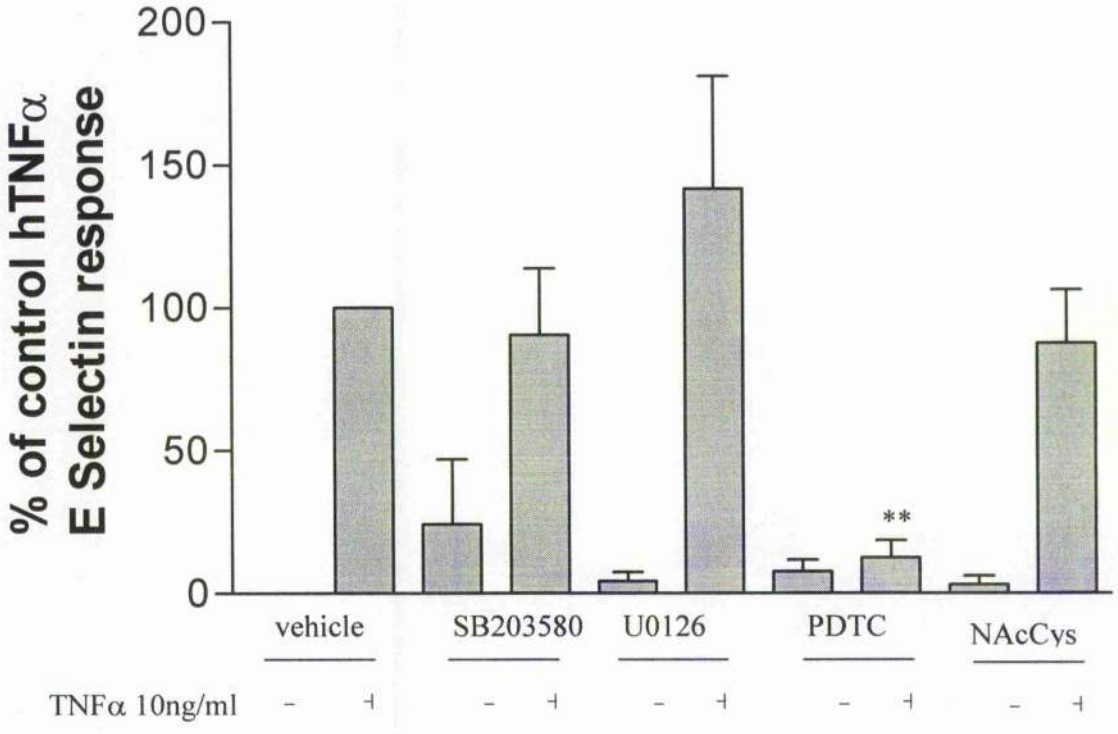


Figure 3.7 Effect of signalling inhibitors on TNFα induced E-Selectin induction

$4x10^4$ HUVECs / well were seeded into a 96 well plate, 24hrs after seeding, cells were pre-treated with either vehicle, 10μM SB203580 (p38 MAPK inhibitor), 100μM U0126 (ERK inhibitor), 100μM PDTC (NFκB inhibitor), 100μM NAcCys (PDTC control) for 30mins then stimulated ± 10ng/ml TNFα for 4hrs. Cells were assessed for E-Selectin induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

** P<0.01 when compared to vehicle + TNFα



involved in arresting leukocytes on activated ECs prior to their migration through the endothelium. Therefore VCAM-1 induction in HUVECs was also examined via cell surface ELISA. To compare the effects of various pro-inflammatory stimuli on VCAM-1 induction, confluent monolayers of HUVECs were treated with LPS, TNFα and IFNγ either on its own or in combination. Figure 3.8 shows that significant VCAM-1 induction is observed with TNFα and to a lesser extent with LPS. Interestingly, IFNγ produced no significant response on its own and had no effect on LPS-mediated induction. Although in the presence of TNFα it appears to suppress the TNFα-mediated induction by nearly 30%, this was found not to be statistically significant. Thus, since it produced the greatest effect, TNFα-mediated VCAM-1 induction was characterised in future experiments.

To ensure the optimum TNFα concentration was utilised for VCAM-1 induction, confluent monolayers of HUVECs were stimulated for 4hrs with increasing concentrations of TNFα (10^{-3}ng/ml-10ng/ml). A significant increase in VCAM-1 induction was first observed with 0.1 ng/ml TNFα this continued to increase with each concentration until a maximum was reached at 10ng/ml, as shown in Figure 3.9. In further experiments the concentration 10ng/ml TNFα was used as it caused the optimal VCAM-1 induction. To determine the stimulation time with TNFα required for optimal VCAM-1 induction, confluent monolayers of HUVECs were stimulated with 10ng/ml TNFα at various time-points over a 24hr period and VCAM-1 induction examined via ELISA as described in section 2.3.4. As seen in Figure 3.10, statistically significant VCAM-1 induction was first observed from 2hrs and increased in a time-dependent manner reaching maximal levels at 8hrs and was sustained for at least 24hrs.

Induction of VCAM-1 in HUVECs has been shown to have some κB dependence (Iademarco et al., 1992). To determine if any other signalling pathways are involved in TNFα-mediated VCAM-1 induction, confluent

Figure 3.8 Effect of different pro-inflammatory stimuli on VCAM-1 induction

$4x10^4$ HUVECs/ well were seeded into a 96 well plate, 24hrs after seeding, cells were stimulated with different pro-inflammatory stimuli (1μg/ml LPS, 10ng/ml TNFα, 10units/ml IFNγ, 1μg/ml LPS + 10units/ml IFNγ, 10ng/ml TNFα+ 10units/ml IFNγ) for 6hrs. Cells were assessed for VCAM-1 induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

*** P<0.001, ** P<0.01, * P<0.05 when compared to vehicle

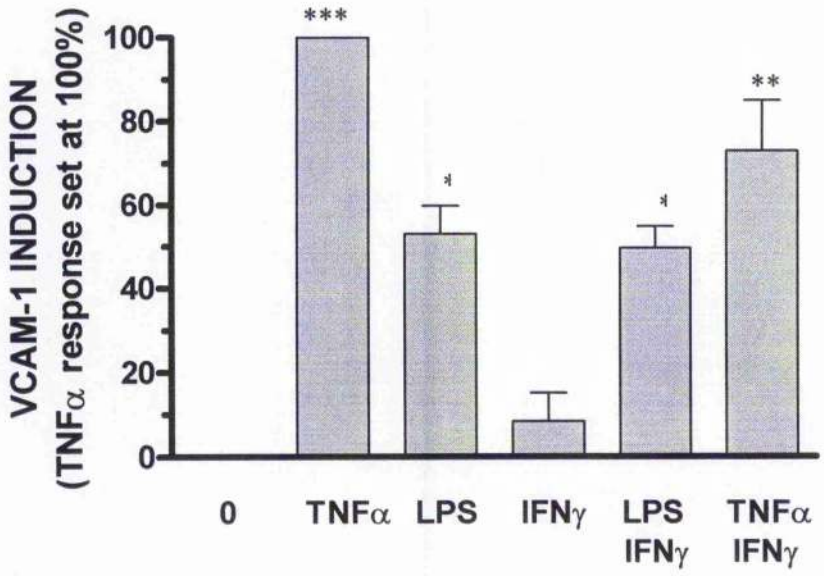


Figure 3.9 Effect of increasing concentrations of TNFα on VCAM-1 Induction

$4x10^4$ HUVECs / well were seeded into a 96 well plate, 24hrs after seeding, cells were stimulated with the indicated concentrations of TNFα for 4hrs. Cells were assessed for VCAM-1 induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

Statistical significance of P<0.001 (***) is observed and sustained from 0.01ng/ml TNFα

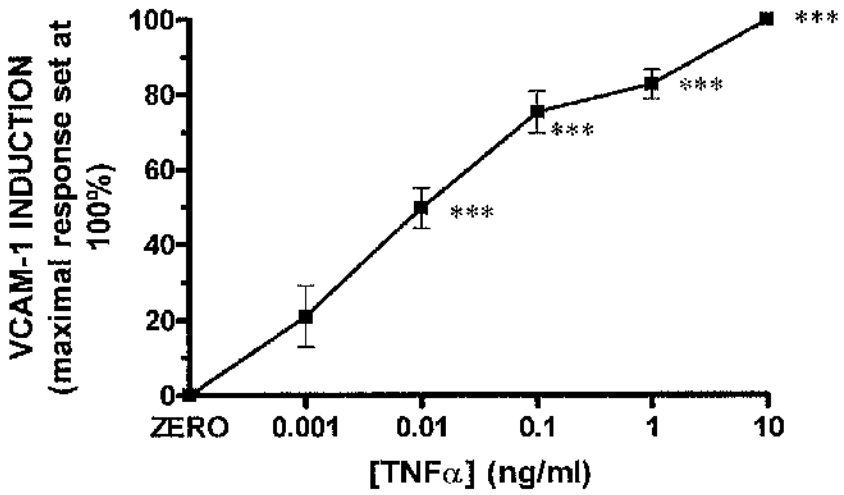
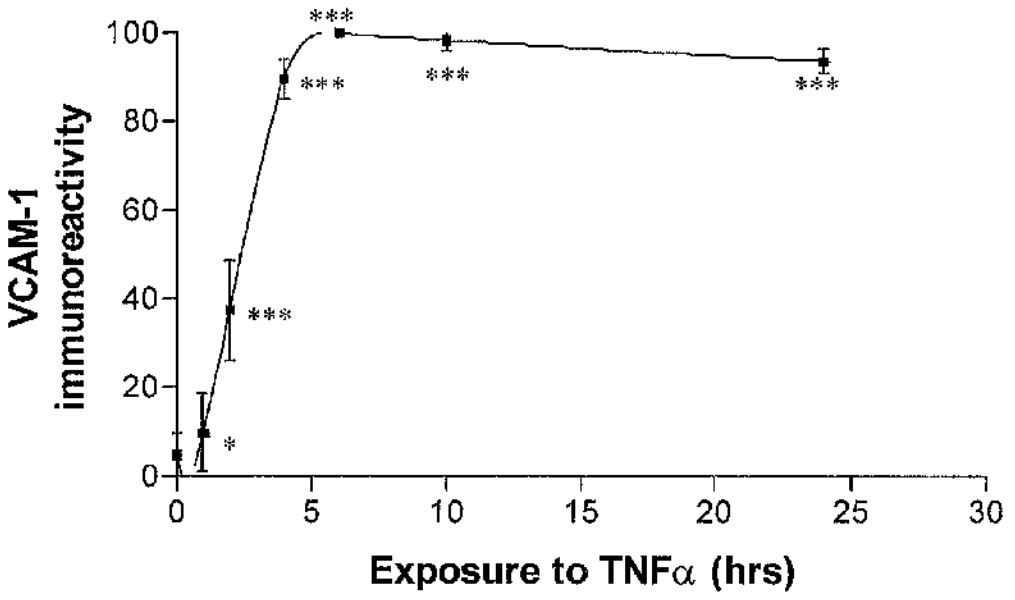


Figure 3.10 Time-course of VCAM-1 induction in response to maximum concentration of TNFα

$4x10^4$ HUVECs / well were seeded into a 96 well plate. 24hrs after seeding, cells were stimulated with 10ng/ml TNFα at the shown time points from 24 hrs. Cells were assessed for VCAM-1 induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

Statistical significance is observed as * = P<0.05 at 2hrs and *** = P<0.001 from 4hrs.



monolayers were pretreated for 30mins with the same panel of signalling inhibitors as used previously (Figures 3.4, 3.5 and 3.7). 4hr TNFα-mediated VCAM-1 induction was examined via ELISA as described in section 2.3.4. Figure 3.11 shows only a slight non-significant decrease in VCAM-1 induction in the presence of NFκB inhibitor, PDTC and also the PKC inhibitor, BIM. This is interesting as VCAM-1 is supposedly a classic κB regulated gene (Ahmed et al., 1998; Iademarco et al., 1992; Lee et al., 2006; Neish et al., 1995). To ensure that the ELISA had not reached saturation, absorbance readings were taken at various times, and the same trend was observed for these readings (data not presented).

It was necessary to ensure adequate A_{2A}AR expression levels were achieved via adenoviral gene transfer before utilising these characterisation data to examine the effect of the A_{2A}AR on TNFα-mediated induction. As mentioned earlier, adenoviral gene transfer was necessary due to the passage-dependent loss of endogenous A_{2A}AR expression observed in HUVECs. Confluent monolayers of HUVECs were infected with 125pfu/cell of either AdA_{2A}AR or control virus AdGFP, which was shown in preliminary experiments to be the optimal MOI required to achieve consistent A_{2A}AR expression. Infected cells were identified under fluorescence at 510nm, by the presence of green fluorescent protein (GFP). Figure 3.12 shows A_{2A}AR gene transfer in HUVECs under phase and fluorescent light with the infection efficiency determined to be ~75%. GFP gene transfer was found to have an infection efficiency of ~75%. To ensure that the AdA_{2A}AR was functional, saturation binding analysis using the specific A_{2A}AR antagonist ^{125}I-ZM241385, was undertaken as described in section 2.3.10. Figure 3.13 shows that AdA_{2A}AR gene transfer facilitates the expression of functional A_{2A}AR.

Having determined the effectiveness of A_{2A}AR gene transfer in HUVECs, its effect on NFκB-regulated COX-2 induction was investigated. Control (Ad GFP) and AdA_{2A}AR infected HUVECs were treated with 10ng/ml TNFα for 8hrs (κB-

Figure 3.11 Effect of signalling inhibitors on TNFα induced VCAM-1 induction.

$4x10^4$ HUVECs /well were seeded into a 96 well plate. 24hrs after seeding, cells were pre-treated with either vehicle, 10μM SB203580 (p38 MAPK inhibitor), 100μM U0126 (ERK inhibitor), 100μM PDTC (NFκB inhibitor), 100μM NAcCys (PDTC control) for 30mins then stimulated ± 10ng/ml TNFα for 6hrs. Cells were assessed for VCAM-1 induction via cell surface ELISA as described in section 2.4.4

The graph shows combined specific binding results for 3 experiments.

No statistical significance was observed between values.

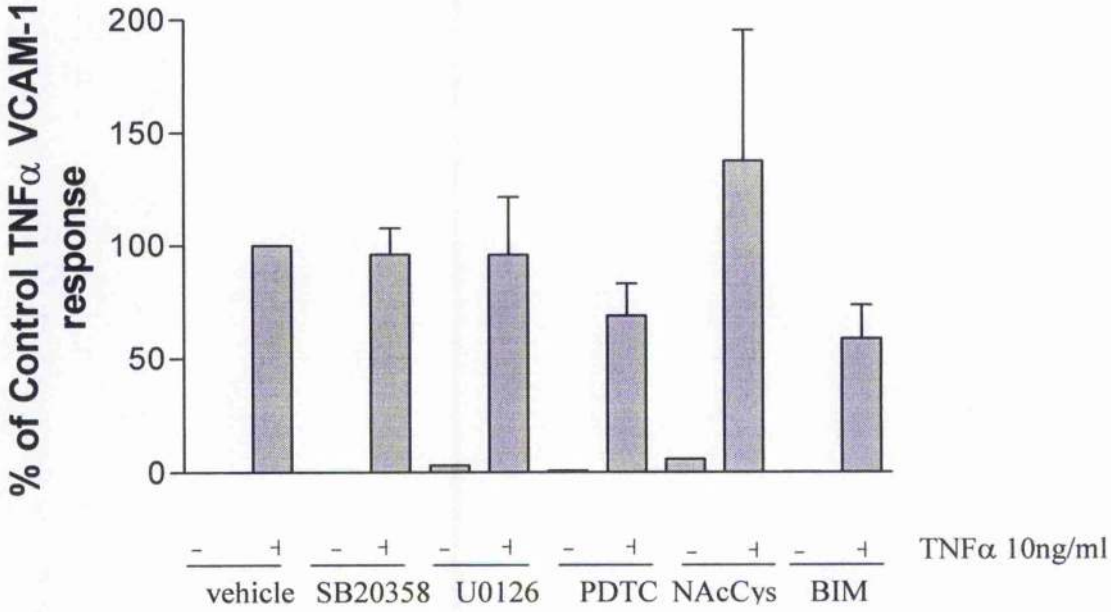


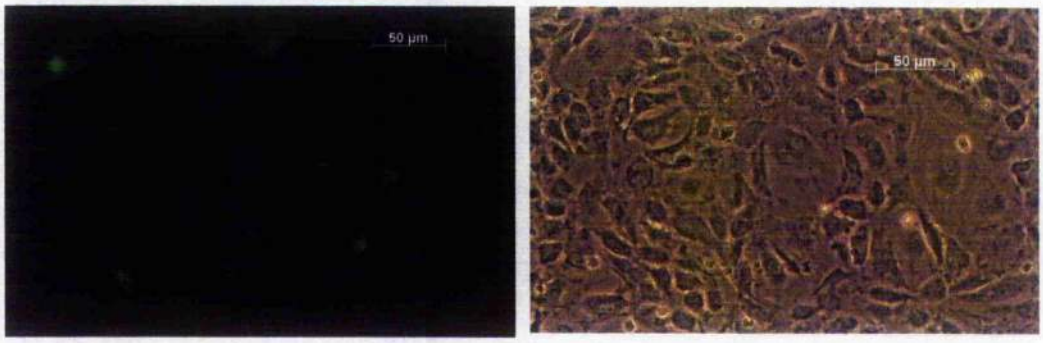
Figure 3.12 $A_{2A}AR$ gene transfer into HUVECS via adenoviral infection

$4x10^5$ HUVECs /well were seeded into 2 x 6 well plates. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells were examined for the presence of GFP via fluorescence microscopy.

Panel A shows $AdA_{2A}AR$ infected HUVECs via fluorescence and phase microscopy.

Panel B shows AdGFP infected HUVECs via fluorescence and phase microscopy.

A $AdA_{2A}AR$



B Ad GFP

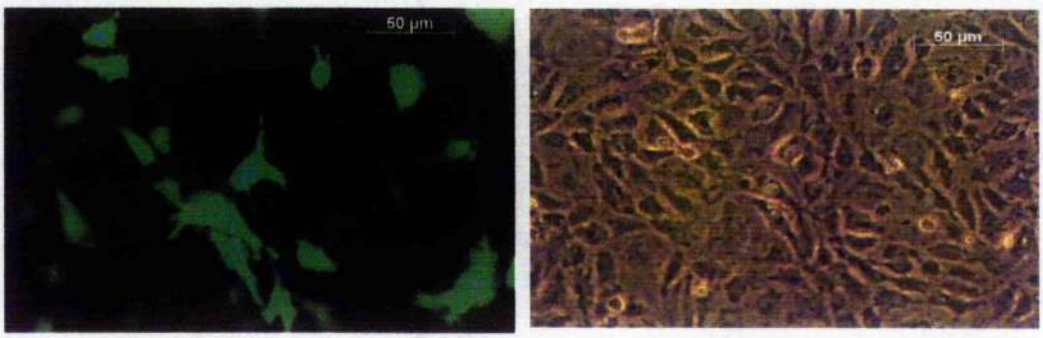
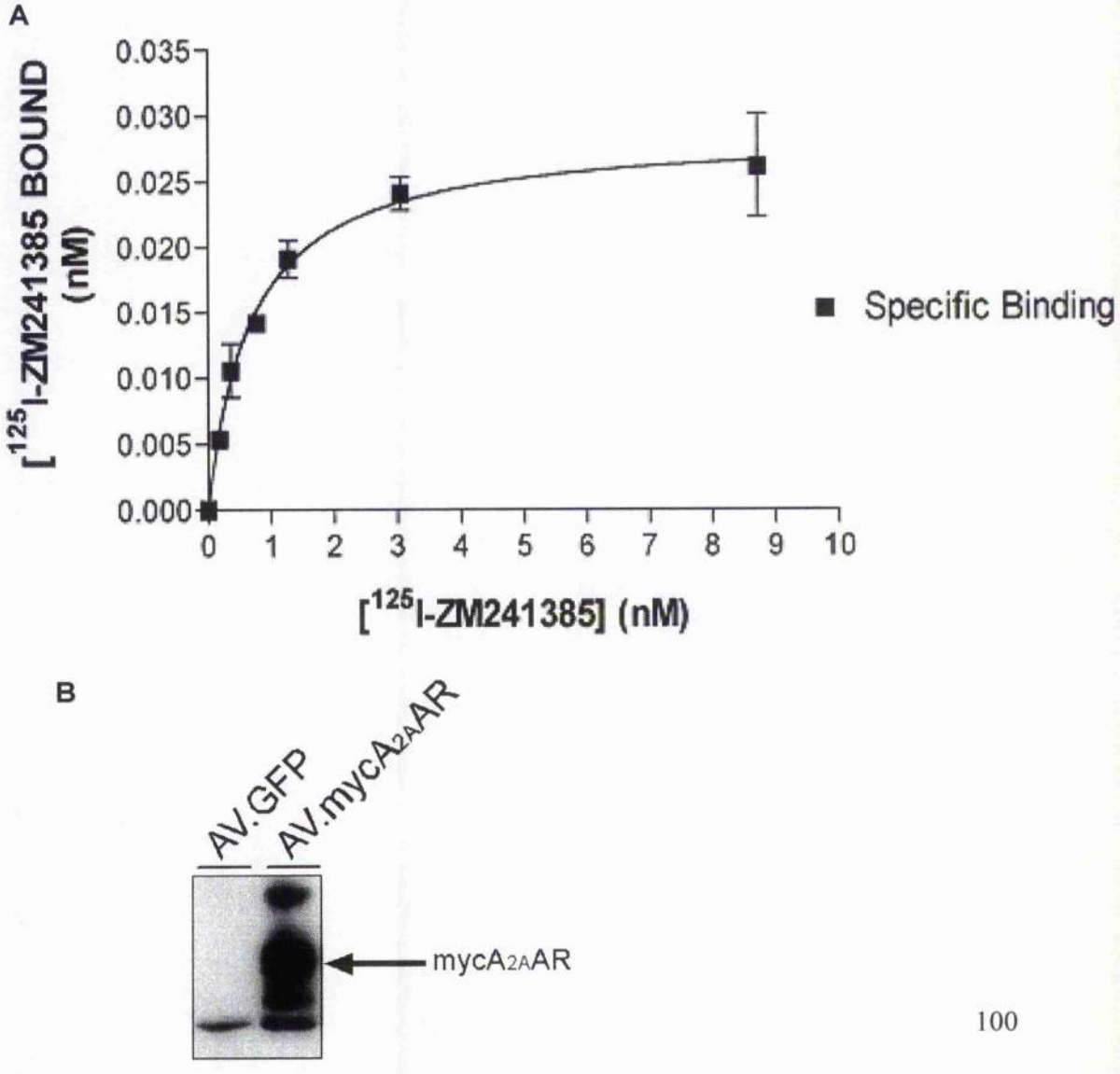


Figure 3.13 Specific binding of $A_{2A}AR$ to selective antagonist radioligand ^{125}I-ZM241385

$8x10^5$ HUVECs /well were seeded into 2 x 10cm dishes. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells membranes were prepared and subjected to saturation binding analysis using the $A_{2A}AR$ selective antagonist radioligand ^{125}I-ZM241385 as described in section 2.4.10.

Panel A is representative of three experiments.

Panel B shows equivalent amounts of the indicated cell lysates fractionated by SDS-PAGE and immunoblotted using anti-myc antibody 9E10.



regulated COX-2 induction detectable at this time; Figure 3.5) in the presence and absence of a maximally effective concentration of A_{2A}AR-selective agonist CGS21680 (Jarvis et al., 1989). Cell lysates were then subjected to SDS-PAGE and immunoblotting with anti-COX-2 antibody. Figure 3.14 indicates that AdA_{2A}AR causes a significant reduction in TNFα-mediated COX-2 induction compared to control GFP-expressing cells. Surprisingly, addition of A_{2A}AR-selective agonist CGS21680 and TNFα to A_{2A}AR-expressing HUVECs actually potentiated the effect of TNFα alone. To initially determine whether these effects could be attributed to regulation of COX-2 by NFκB, Ad GFP- and AdA_{2A}AR-infected HUVECs were treated as Figure 3.14 for 24hrs, a time-point at which NFκB had previously been shown not to regulate COX-2 induction (Figure 3.4). Figure 3.15 shows that no significant change in COX-2 induction was observed in the A_{2A}AR expressing cells when compared to the GFP-expressing control cells. This suggests that the observed effects (Figure 3.14) in A_{2A}AR-expressing HUVECs are mediated at least in part by NFκB regulation.

The A_{2A}AR is predominately coupled to Gs, leading to the activation of adenylyl cyclase and elevation of intracellular cAMP (Olah, 1997). Thus, to investigate whether the changes in COX-2 induction could be reproduced by increasing intracellular cAMP, control uninfected HUVECs were pre-treated for 30mins with either rolipram (phosphodiesterase (PDE) 4-selective inhibitor; Zhu et al., 1998), forskolin (direct adenylyl cyclase catalytic activator; de Souza et al., 1983), or A_{2A}AR-selective agonist CGS21680 and then stimulated with a submaximal concentration of TNFα (1ng/ml). Cell lysates were then subjected to SDS-PAGE and immunoblotting with anti-COX-2 antibody. Figure 3.16 indicates that forskolin either on its own or in conjunction with rolipram potentiates TNFα-mediated COX-2 induction. To identify whether increasing cAMP was sufficient to induce COX-2 in the absence of TNFα, HUVECs were treated with a combination of rolipram and forskolin at various time-points over a 48hr period. Rolipram and forskolin treatment appears to cause a transient increase in COX-2 induction, as shown in Figure 3.17, with initial induction

Figure 3.14 Effect of Ad A_{2A}AR Gene Transfer on COX-2 induction by TNFα

$4x10^5$ HUVECs /well were seeded into 2 x 6 well plates. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His A_{2A}AR or Ad GFP. 48hrs post infection, cells were treated with ± 5μM CGS21680 (A_{2A}AR-selective agonist), ±10ng/ml TNFα for 8hrs. Cells were harvested, lysed, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.
The graph shows the combined results for 3 experiments and the blots are representative of one experiment.

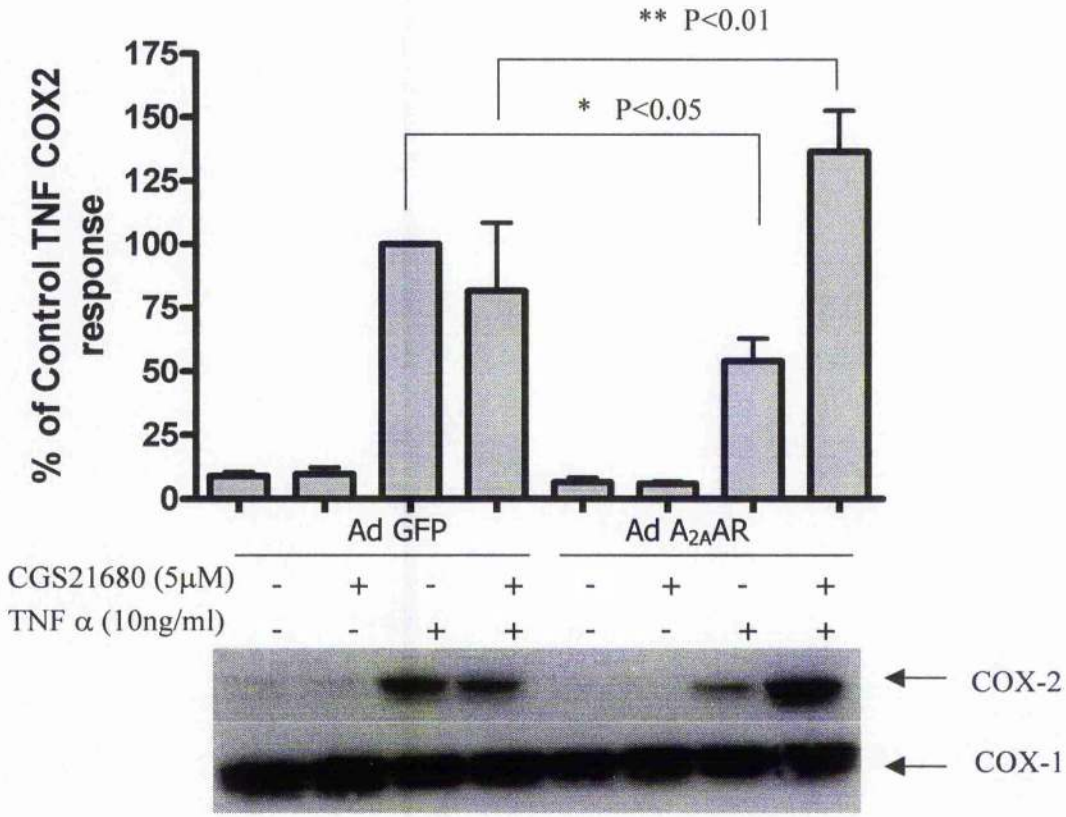


Figure 3.15 Effect of Ad A_{2A}AR Gene Transfer on COX-2 induction by TNFα

$4x10^5$ HUVECs /well were seeded into 2 x 6 well plates. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His A_{2A}AR or Ad GFP. 48hrs post infection, cells were treated with ± 5μM CGS21680 (A_{2A}AR-selective agonist), ±1ng/ml TNFα for 24hrs. Cells were harvested, lysed, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.
The graph shows the combined results for 3 experiments and the blots are representative of one experiment.
No statistical significance observed

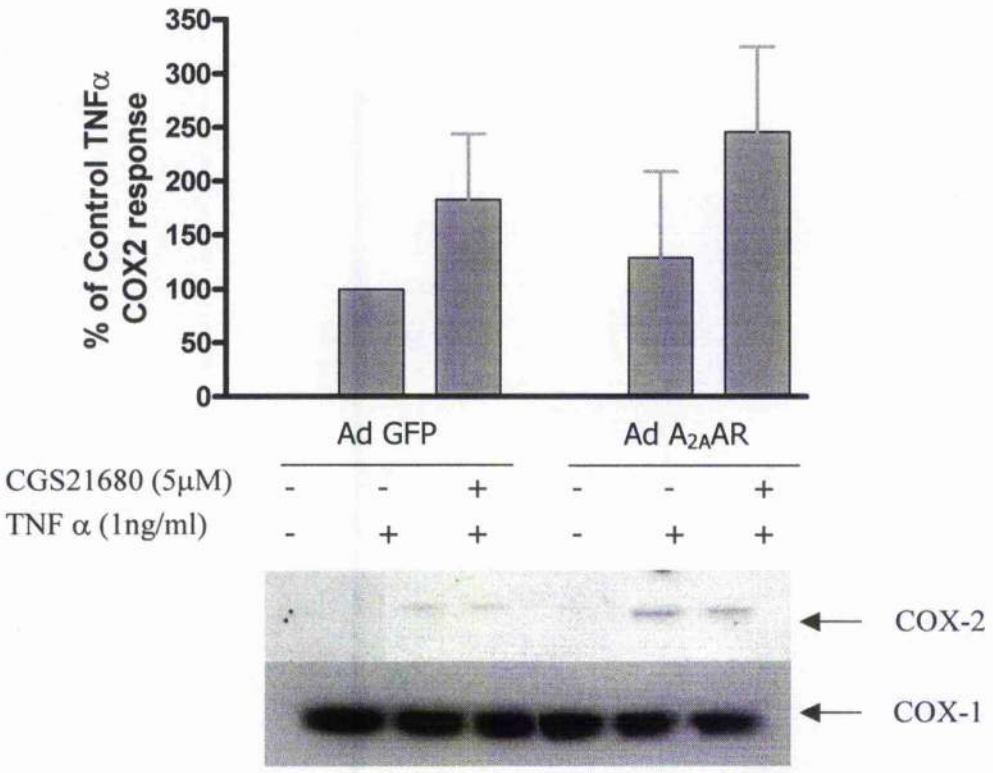


Figure 3.16 Effects of cAMP elevating agents on COX-2 induction in the presence of TNFα

$4x10^5$ HUVECs / well were seeded into 2 x 6well plate. 24hrs after seeding, cells were pretreated for 30mins with vehicle, 10μM rolipram (PDE inhibitor), 10μM forskolin (adenyl cyclase activator), 10μM rolipram +10μM forskolin, 5μM CGS21680, then stimulated with a sub maximal concentration of TNFα (1ng/ml) for 24hrs. Cells were harvested, lysed, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.

The graph shows combined results for 3 experiments and the blots are representative of one experiment.

*** = P<0.001, * = P<0.05 when compared with vehicle + TNFα

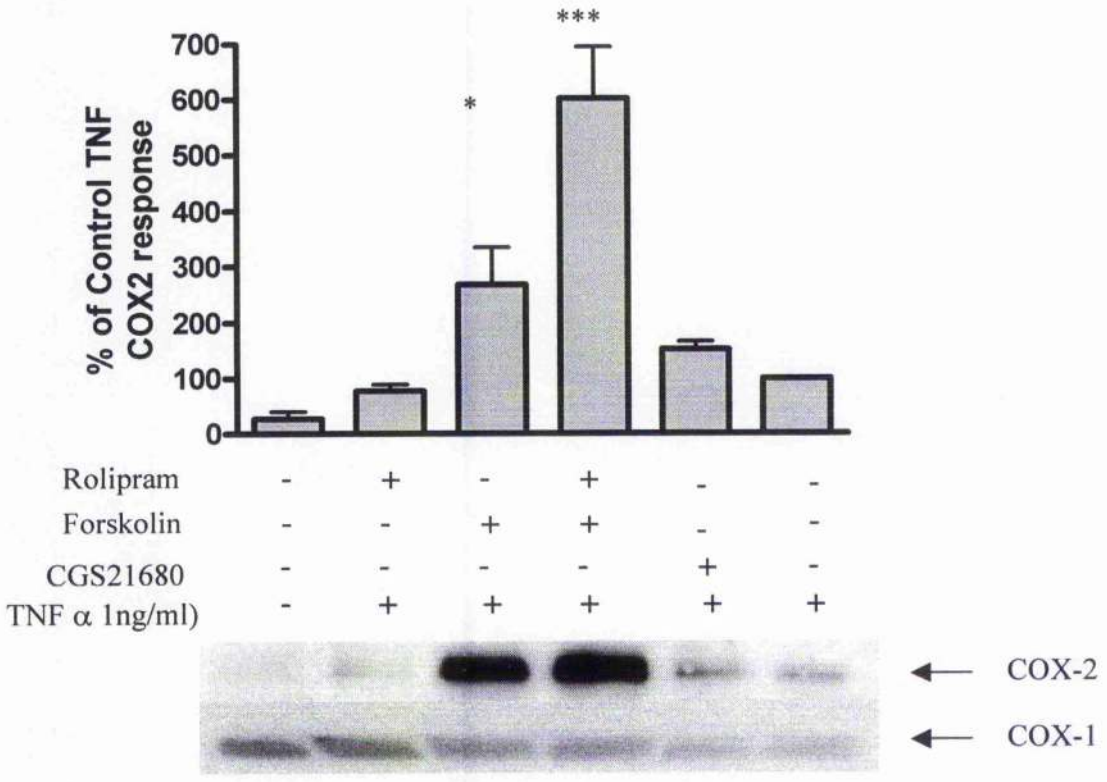
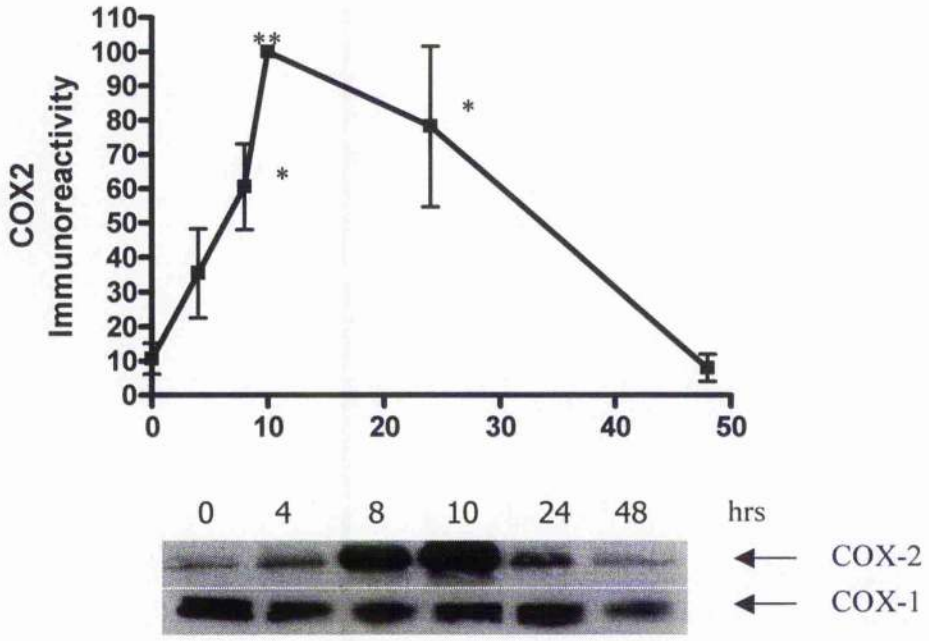


Figure 3.17 Time course of the effect of rolipram and forskolin on COX-2 induction in the absence of TNFα

$4x10^5$ HUVECs / well were seeded into a 6 well plate. 24hrs after seeding, cells were treated with combination of 10μM rolipram and 10μM forskolin at 0, 4, 8, 10, 24 and 48hrs. Cells were harvested, lysed, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3. The graph shows combined results for 3 experiments and the blots are representative of one experiment.

Statistical significance is observed as * = P<0.05 at 8hrs and 24hrs and ** = P<0.01 at 12 hrs.



observed at 4hrs, maximal response observed at 10hrs and returned to basal levels after 48hrs. Thus increasing intracellular cAMP levels is sufficient to promote COX-2 induction.

There are two possible methods by which the A_{2A}AR is able to achieve this. Firstly that the A_{2A}AR activates two downstream pathways whereby transient A_{2A}AR expression is able to inhibit p65 nuclear accumulation and active A_{2A}AR signalling increases intracellular cAMP resulting in an upregulation of CREB target proteins. The second possibility is that there is a threshold of cAMP concentration. Below this cAMP concentration threshold, the effect of the A_{2A}AR is inhibitory causing an inhibition of COX-2 induction whereas above this threshold the effect of the A_{2A}AR is stimulatory causing a potentiation of COX-2 induction due to the upregulation of CREB. To identify if there is a threshold concentration of cAMP at which COX-2 induction was potentiated in the presence of TNFα, HUVECs were pre-treated with differing concentrations of forskolin and then stimulated with TNFα for 8hrs. Consistent with this hypothesis, it was observed that low concentrations (0.1μM) of forskolin suppressed COX-2 induction whilst at higher concentrations enhanced COX-2 induction (Figure 3.18). Thus, low intracellular cAMP levels inhibited COX-2 induction while high cAMP levels resulted in a strong COX-2 induction that is able to counteract the inhibitory effect on NFκB.

To determine whether the same complex pattern of regulation was observed with other κB-regulated genes, comparative analysis of E-selectin by cell surface ELISA revealed that while induction was suppressed by A_{2A}AR, the suppression was more pronounced in the presence of CGS21680 (Figure 3.19). This correlated with the effects of treating control uninfected cells with cAMP-elevating stimuli (Figure 3.20) which inhibited TNFα-mediated E-selectin induction. Thus, it can be concluded that TNFα-mediated E-selectin induction is solely dependent on NFκB activation and is further inhibited by cAMP under these conditions.

Figure 3.18 Effect of Forskolin Dose Response on COX-2 induction

$4x10^5$ HUVECs / well were seeded into 6 well plate. 24hrs after seeding,cells were pretreated for 30mins with vehicle or with concentrations of forskolin (adenylyl cyclase activator;) ranging from 10^{-10} to 10^{-5}, then stimulated with TNFα (10ng/ml) for 8hrs. Cells were harvested, equalised for protein concentration and assessed for COX-2 and then COX-1 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.

The graph shows combined results for 3 experiments and the blots are representative of one experiment.

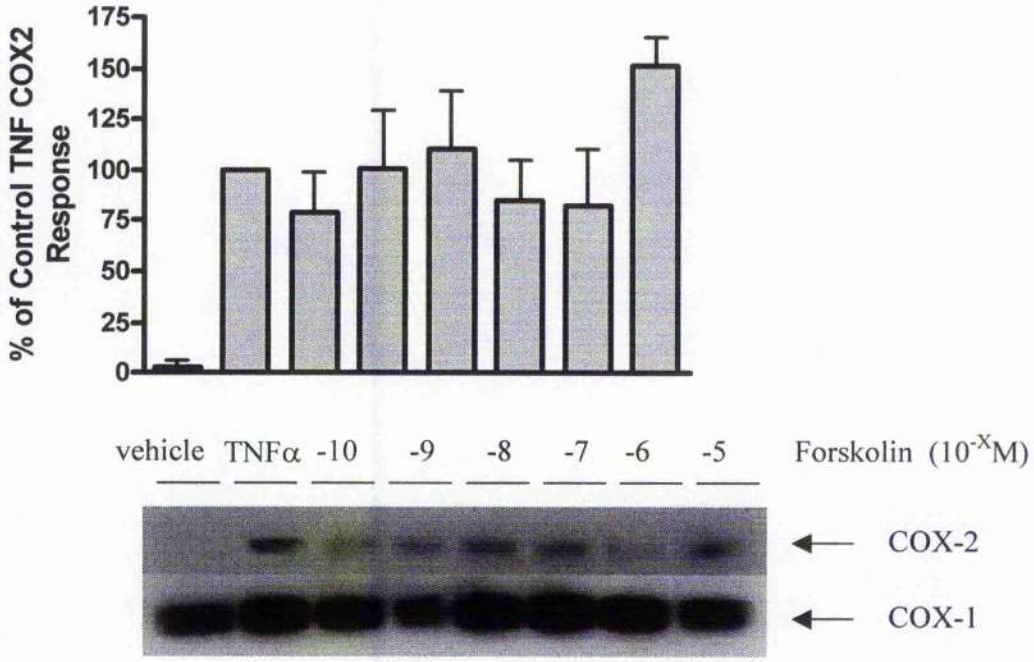


Figure 3.19 Effect of Ad A_{2A}AR gene transfer on E-Selectin induction by TNFα.

$2x10^4$ HUVECs / well were seeded into 96 well pate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His A_{2A}AR or Ad GFP. 48hrs post infection, cells were treated with ± 5μM CGS21680 (A_{2A}-selective agonist), ±10ng/ml TNFα for 4hrs. Cells were assessed for E-Selectin induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

Statistical significance is observed as * = P<0.05.

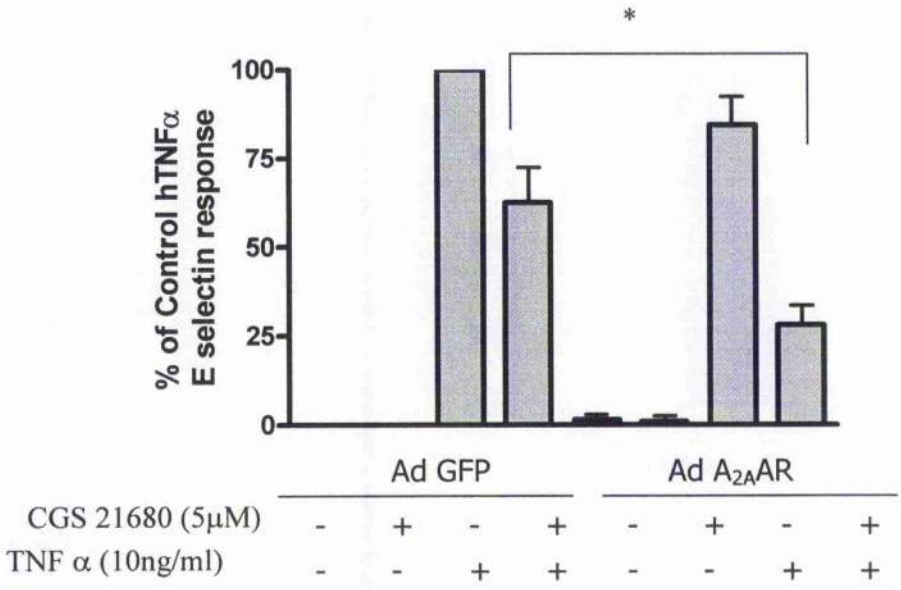
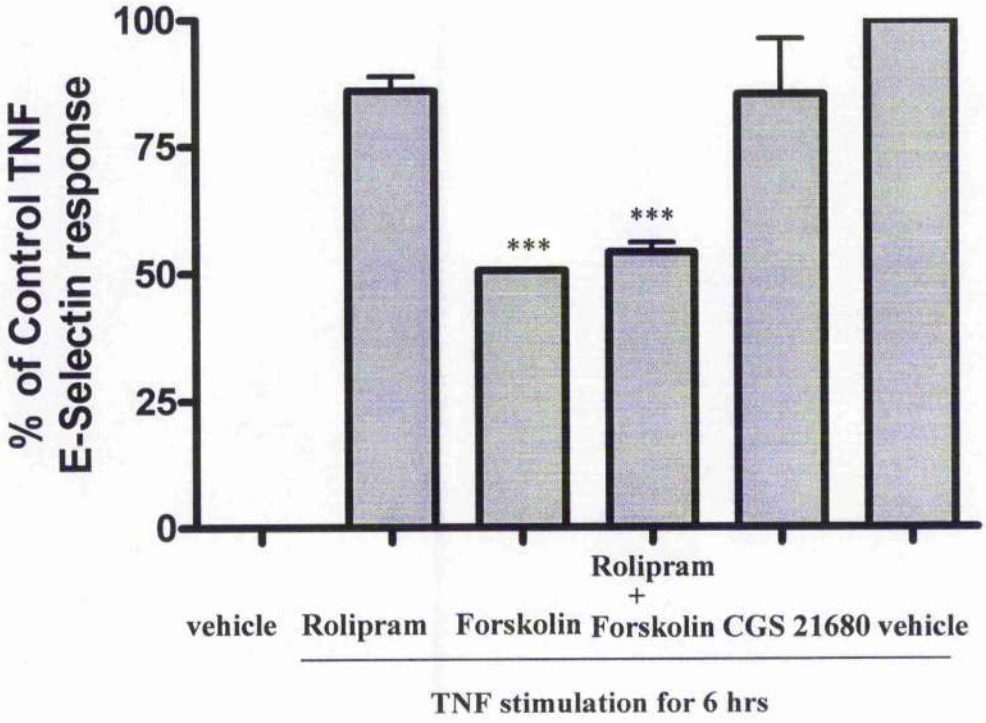


Figure 3.20 Effects of cAMP elevating agents on E-Selectin induction by TNFα

$4x10^4$ HUVECs / well were seeded into 96 well plate. 24hr after seeding, cells were pretreated for 30mins with vehicle, 10μM rolipram (PDE inhibitor), 10μM forskolin (adenylyl cyclase activator), 10μM rolipram +10μM forskolin, 5μM CGS21680, then stimulated with a sub maximal concentration of TNFα (1ng/ml) for 4hrs. Cells were assessed for E-Selectin induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

*** = P<0.001 when compared to vehicle + TNFα



In the case of VCAM-1 the results were distinct from those of E-selectin and COX-2. A_{2A}AR gene transfer had no effect on TNFα-mediated VCAM-1 induction in the presence or absence of CGS21680 (Figure 3.21). Also in control cells, sub-maximal TNFα-mediated VCAM-1 induction was not affected positively or negatively by cAMP-elevating stimuli (Figure 3.22). Although it has been reported that VCAM-1 induction can be reduced via increased cAMP levels via the A_{2A}AR (Bouma et al., 1996) this was not reproducible in our hands.

COX-2 is the rate-determining enzyme for the production of biologically active prostanoids (Caughey et al., 2001). To determine whether the potentiation in COX-2 induction, observed in A_{2A}AR expressing cells in the presence of CGS21680 and TNFα, caused any downstream effect on COX-2-regulated prostaglandin E_2 and prostacyclin concentrations, conditioned media from treated samples samples were examined via ELISA as described in section 2.4.8. There was no difference in prostaglandin E_2 generation in TNFα-stimulated A_{2A}AR-expressing cells when compared with control GFP-expressing cells. However, the presence of CGS21680 and TNFα caused a significant increase in prostaglandin E_2 generation (Figure 3.23) in A_{2A}AR-expressing cells when compared to TNFα-stimulated A_{2A}AR-expressing cells or control GFP-expressing cells. Samples of the same conditioned medium were also assayed for prostacyclin via the presence of the stable prostacyclin breakdown product, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). As shown in Figure 3.24, a slight reduction in 6-keto-$PGF_{1\alpha}$ generation was observed in TNFα-stimulated A_{2A}AR expressing HUVECs when compared to control cells whilst combined treatment with CGS21680 and TNFα caused an increase in 6-keto-$PGF_{1\alpha}$ production in both A_{2A}AR-expressing cells and control cells. However this increase was only statistically significant in the A_{2A}AR-expressing cells when compared to TNFα-stimulation alone. These data correlates with the increase in COX-2 induction following the same treatment in A_{2A}AR-expressing cells which correlated with

Figure 3.21 Effect of $A_{2A}AR$ gene transfer on TNFα-stimulated VCAM-1 induction

$2x10^4$ HUVECs / well were seeded into 96 well pate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells were treated with ± 5μM CGS21680 (A_{2A}-selective agonist), ±10ng/ml TNFα for 6hrs. Cells were assessed for VCAM-1 induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

No statistical significance was observed between values.

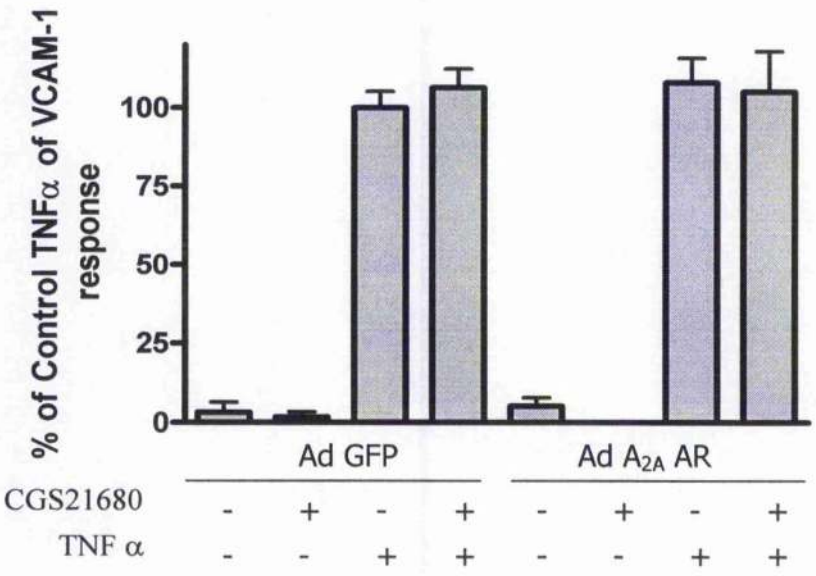


Figure 3.22 Effects of cAMP elevating agents on VCAM-1 induction in the presence of TNFα

$4x10^4$ HUVECs / well were seeded into 96 well plate. 24hrs after seeding, cells were pretreated for 30mins with vehicle, 10μM rolipram (PDE inhibitor), 10μM forskolin (adenylyl cyclase activator), 10μM rolipram + 10μM forskolin, 5μM CGS21680, then stimulated with a sub maximal concentration of TNFα (1ng/ml) for 6hrs. Cells were assessed for VCAM-1 induction via cell surface ELISA as described in section 2.4.4.

The graph shows combined specific binding results for 3 experiments.

No statistical significance was observed between values.

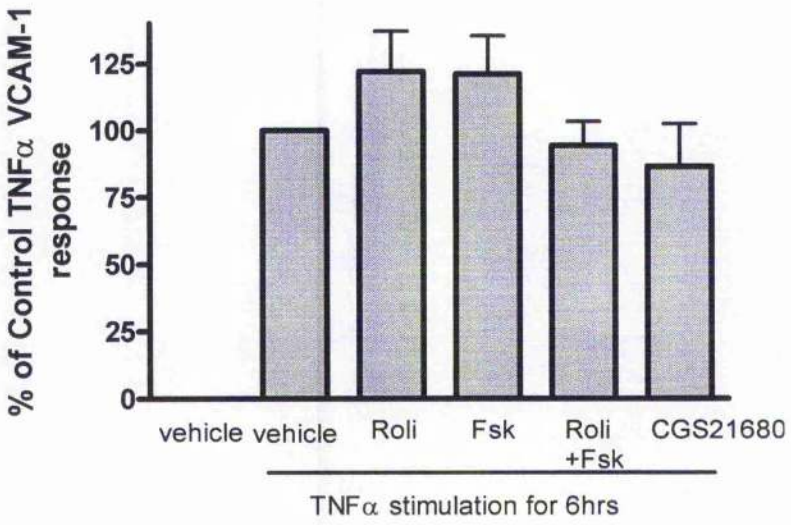


Figure 3.23 Effect of Ad A_{2A}AR gene transfer on Prostaglandin E_2 accumulation

$2x10^5$HUVECs / well were seeded into 2 x 24 well plate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His A_{2A}AR or Ad GFP. 48hr post infection, cells were treated with ± 5μM CGS21680 (A_{2A}-selective agonist), ±10ng/ml TNFα for 8hrs. Spent medium was collected and analysed for prostaglandin E_2 concentration via ELISA as described in section 2.4.8.

The graph represents data from three experiments

Statistical significance of $P<0.05$ (*) observed between indicated values.

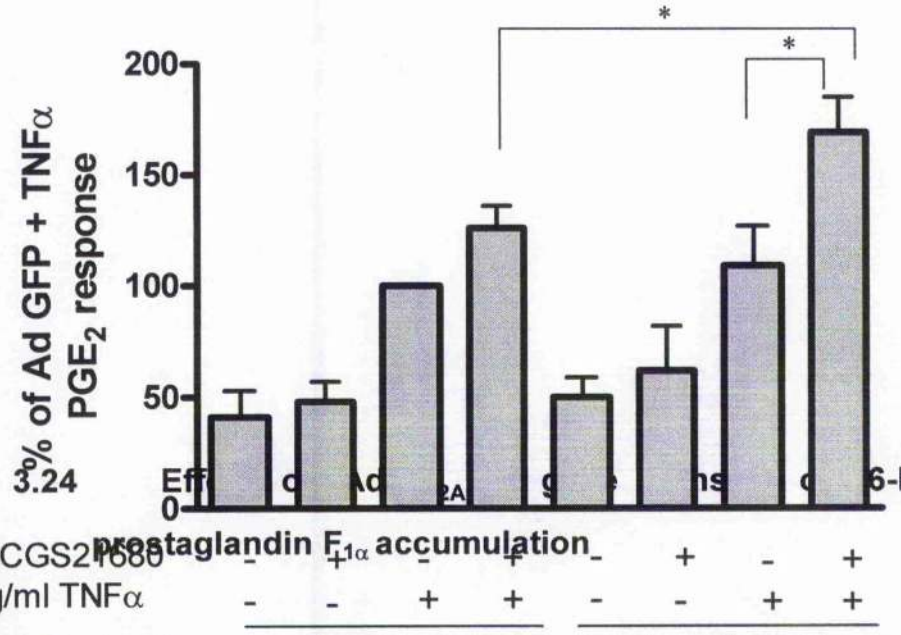
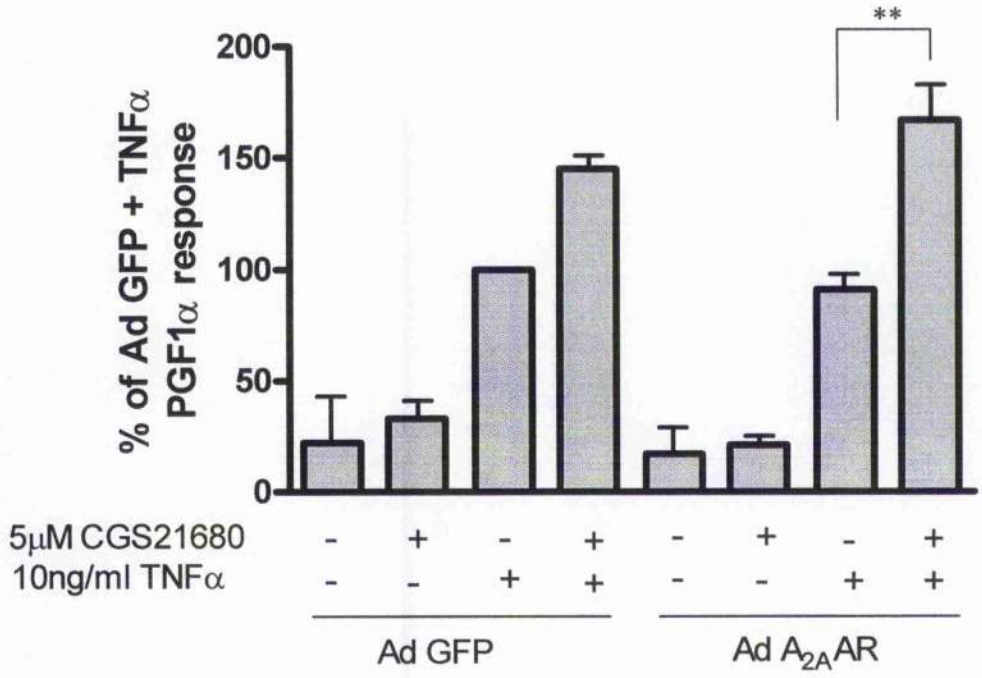


Figure 3.24 Effect of Ad A_{2A}AR gene transfer on 6-keto-prostaglandin $F_{1\alpha}$ accumulation

$2x10^5$HUVECs / well were seeded into 2 x 24 well plate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad myc-His A_{2A}AR or Ad GFP. 48hr post infection, cells were treated with ± 5μM CGS21680 (A_{2A}-selective agonist), ± 10ng/ml TNFα for 8hrs. Spent medium was collected and analysed for 6-keto-prostaglandin $F_{1\alpha}$ concentration via ELISA as described in section 2.4.8.

The graph represents data from three experiments run on one ELISA plate. Statistical significance of P<0.01 (**) observed between indicated vaules



COX-2 mediating the production of prostaglandin E_2 and prostacyclin from arachidonic acid.

To identify whether cAMP elevating agents could also increase prostaglandin E_2 and prostacyclin production, control cells were treated with cAMP elevating agents over a 24hr time-period. Figure 3.25 shows an approximate 50% increase in prostaglandin E_2 production following 8hr TNFα treatment which is sustained until 24hrs. Figure 3.26 shows that this trend was also observed in 6-keto-$PGF_{1\alpha}$ production. However the increase in both 6-keto-$PGF_{1\alpha}$ and prostaglandin E_2 production proved to not to be statistically significant. It must be noted that although these experiments were undertaken in triplicate all samples were analysed on the same ELISA plate and significance may be observed if repeated.

The regulation of mRNA stability is an important factor in modulating gene expression, in particular for transiently expressed genes that require tightly controlled mRNA levels. For different cytokines such as TNFα (Caput et al., 1986) and mRNAs of inducible enzymes such as COX-2 (Dean et al., 2002), modulating the decay rate involves adenylate uridylate-rich elements (AREs) which often consist of one or more copies in the 3'-untranslated region (3'UTR) (Chen and Shyu, 1995). COX-2 mRNA has been shown to be stabilised by specific inflammatory stimuli which target the ARE through the p38 mitogen-activated protein kinase (MAPK) signalling pathway (Dean et al., 1999; Ridley et al., 1998) (Kumar et al., 2003). This provides an explanation for the inhibition of COX-2 induction observed following pre-treatment with p38 MAP kinase inhibitor SB203580 in Figure 3.4 and 3.5. To identify any possible role A_{2A}AR gene transfer has on COX-2 mRNA levels which would explain the observed changes in COX-2 protein levels, quantitative PCR analysis of COX-2 mRNA levels was performed as described in Section 2.3.7. Identification of the optimal time-point for examining the effects of A_{2A}AR gene transfer was undertaken by a 24hr time-course of TNFα stimulation in control cells. RNA was extracted and

Figure 3.25 Effect of Forskolin and Rolipram treatments on Prostaglandin E_2 generation

$2x10^5$ HUVECs / well were seeded into 2 x 24 well plate. 24hrs after seeding, cells were treated with combination of 10μM rolipram +10μM forskolin at 0, 8, 10 and 24hrs. Spent medium was collected and analysed for prostaglandin E_2 concentration via ELISA as described in section 2.4.8.

The graph represents data from three experiments run on one ELISA plate.

No statistical significance observed

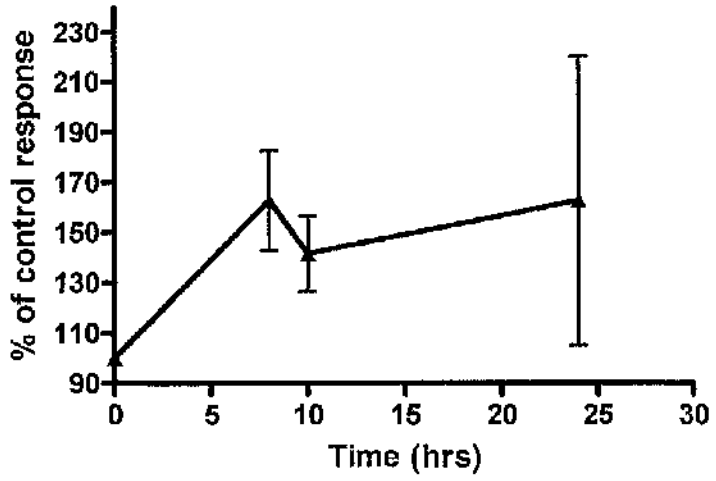
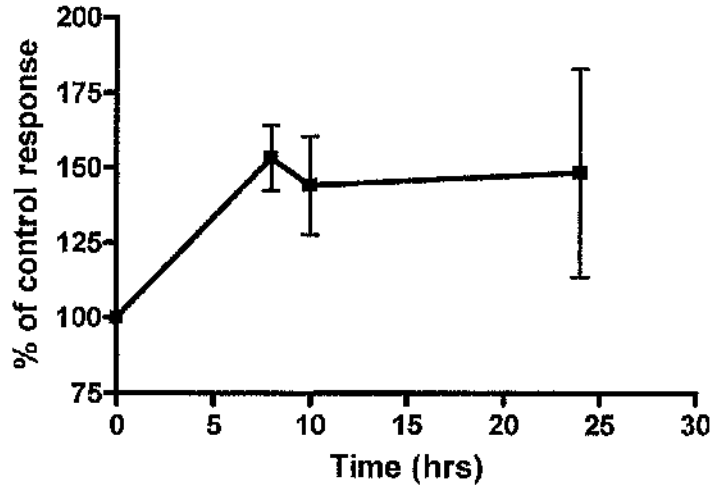


Figure 3.26 Effect of Forskolin and Rolipram treatments on 6-keto-prostaglandin $F_{1\alpha}$ generation

$2x10^5$HUVECs / well were seeded into 2 x 24 well plate. 24hrs after seeding, cells were treated with combination of 10μM rolipram +10μM forskolin at 0, 8, 10 and 24hrs. Spent medium was collected and analysed for 6-keto-prostaglandin $F_{1\alpha}$ concentration via ELISA as described in section 2.4.8.
The graph represents data from three experiments
No statistical significance observed



cDNA generated using random primers which was then examined by q-RT-PCR using *cox-2* and the housekeeping gene, *gapdh* primers as described in section 2.3.8. Initial examination suggested that the optimal accumulation of COX-2 mRNA occurred following 6hr of TNFα treatment, therefore this time-point was chosen to examine if A_{2A}AR-gene transfer has any effect on COX-2 mRNA levels. Control and A_{2A}AR expressing cells were treated ± TNFα and ± CGS21680 for 6hrs, RNA extraction and examination by qRT-PCR as sections 2.3 6 - 2.3.8. However consistency between reaction triplicates and reaction plates could not be attained. To determine whether this inconsistency was due to primer-dimer formation or the amplification of introns, PCR was undertaken with the *cox-2* and *gapdh* primers using either genomic DNA or cDNA generated from TNFα-treated HUVECS as a template. Figure 3.27 shows that neither the *cox-2* primers or *gapdh* primers produced PCR product from genomic DNA nor generated any primer-dimer formation. This suggests that the observed inconsistencies with the qRT-PCR cannot be explained by inconsistencies with the primers. Examination of the experimental technique provided no further answers for the inconsistencies between triplicates as the PCR mixture was obtained from two master-mixes.

3.3 Discussion

The A_{2A}AR has been identified as a protective anti-inflammatory receptor protein not only by numerous pharmacological studies (Linden, 2001) but also from characterisation of inflammatory responses in mice in which both copies of the A_{2A}AR gene have been deleted (Ohta and Sitkovsky, 2001). Previous work undertaken in our lab has shown that increasing A_{2A}AR gene expression suppresses the induction of TNFα-mediated E-selectin induction by more than 70% in HUVECs (Sands et al., 2004). This was shown to occur by A_{2A}AR gene expression suppressing NFκB activation in response to stimuli such as TNFα and LPS by reducing the nuclear accumulation of p50/p65 heterodimers (Sands et al., 2004). To assess in greater detail potential functional consequences of

Figure 3.27 Examination of PCR primers for qRT-PCR

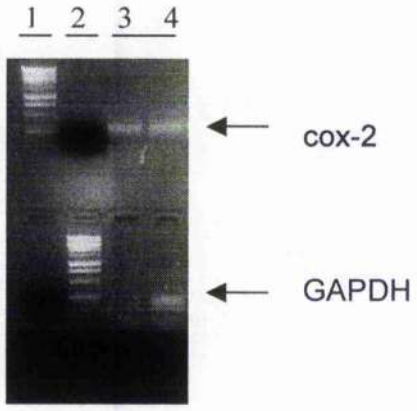
8 x 10^5 HUVECs was seeded out into 1 x 10cm plates. 24hrs after seeding cells were treated with 10ng TNFα for 6hrs. Cells were harvested into 1ml tri reagent and RNA extracted as described in section 2.3.6. 2ng RNA was then used to generate cDNA as described in section 2.3.6. 1ng of cDNA and 1ng of genomic DNA was then used as templates in PCR reactions with *cox-2* or *gapdh* primers and Taq polymerase. The PCR program was run as section 2.3.7. 5ul of PCR product was then run on a 2% agarose gel with 1kb markers.

cox-2 primers-

Lane 1 1kb Marker, Lane 2 genomic DNA, Lane 3 cDNA, Lane 4 cDNA + DMSO

gapdh primers –

Lane 1 empty, Lane 2 1kb Marker, Lane 3 genomic DNA, Lane 4 cDNA

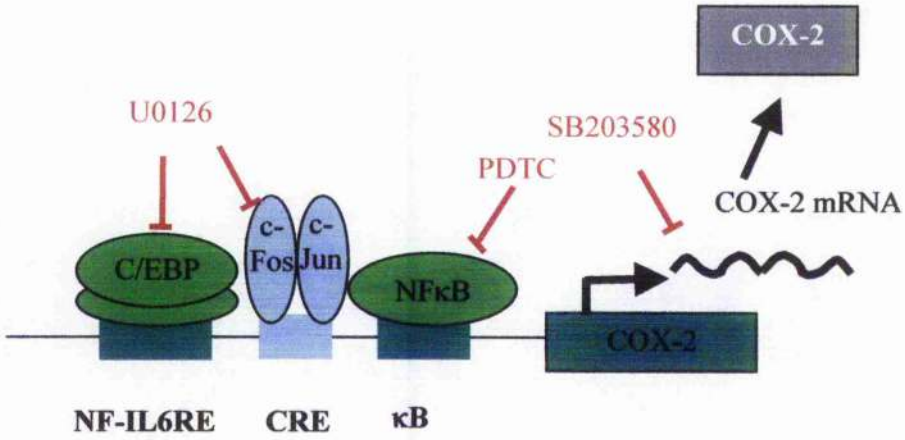


this phenomenon, we have compared the effects of A_{2A}AR gene transfer in HUVECs on the accumulation of three important EC gene products known to be controlled at least in part by NFκB: COX-2, E-selectin and VCAM-1.

Characterisation of COX-2 induction in response to TNFα revealed that while sustained induction (24hr) was solely dependent on activation of p38 MAP kinase, earlier time-points at which induction was first detectable (8hr) were also sensitive to inhibition of the NFκB pathway. These findings are consistent with what is currently known about the promoter region of the human *COX-2* gene (Figure 3.28) which has been shown to contain several potential transcription regulatory elements, including an NF-IL6 motif, two AP-2 sites, three Sp1 sites, two NFκB sites, a cAMP response element (CRE) motif and an E-box (Tanabe and Tohnai, 2002). There are many consensus *cis*-elements in the 5'-flanking region regulating the transcription of the COX-2 gene. However only a limited number of elements, namely the CRE site, NF-IL6 motif, NFκB site and the E-box are known to be involved in the regulation of COX-2 gene expression (Tanabe and Tohnai, 2002). The CRE site has been identified as one of the most essential regulatory elements in the COX-2 promoter region. Homo-or hetero-dimers of c-fos, c-jun, ATF family members and the cAMP response element binding protein (CREB) can bind to this element and activate COX-2 expression (Inoue et al., 1995; Nakabeppu et al., 1988; Rauscher et al., 1988; van Dam et al., 1993). Inoue et al. (1995) reported that destruction of both the CRE and NF-IL6 regulatory elements markedly reduced COX-2 expression by 75% in human endothelial cells following treatment with LPS. However destruction of either the NF-IL6 motif or the CRE caused 40% and 10% reductions respectively (Inoue et al., 1995). TNFα induces activation and binding of NFκB to two separate κB enhancer elements on the core promoter region and mutation of either enhancer element greatly reduces the TNFα-stimulated transcriptional activity (Saunders et al., 2001), suggesting that CRE element is regulated by a different mechanism. This is consistent with what we

Figure 3.28 Schematic of *cox-2* promoter

Regulatory elements in the human COX-2 promoter indicating points of interaction by p38 MAPK inhibitor, SB203580, ERK inhibitor, U0126 and NFκB inhibitor PDTC.



have also shown, namely the COX-2 induction by TNFα depends at least in part on NFκB activation and binding.

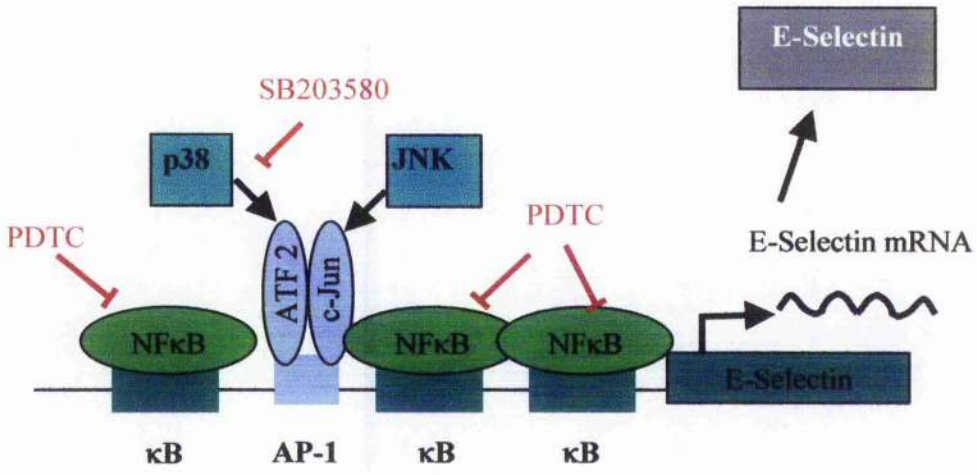
At 8hrs, A_{2A}AR gene transfer alone was sufficient to reduce TNFα-stimulated COX-2 induction compared with control cells. Surprisingly, the addition of the A_{2A}AR selective agonist CGS21680 to A_{2A}AR-expressing HUVECs actually reversed the effect seen with receptor alone. A_{2A}AR activation elevates intracellular cAMP levels in HUVECs via interaction with G_s and stimulation of adenylyl cyclase (Klinger et al., 2002). Therefore exposing A_{2A}AR-expressing HUVECs to CGS21680 will increase the intracellular levels of cAMP. To determine if the potentiation of COX-2 induction observed in TNFα-stimulated A_{2A}AR expressing HUVECs was due to increasing levels of cAMP, control HUVECs were pre-treated with various cAMP elevating agents; PDE inhibitor (rolipram), adenylyl cyclase stimulator (forskolin) and CGS21680, before treatment with submaximal concentration of TNFα. We observed a five-fold increase in COX-2 induction following combined pre-treatment with both PDE inhibitor and adenylyl cyclase stimulator when compared to TNFα alone. This suggests that increasing concentrations of cAMP can explain the potentiation of COX-2 induction observed in A_{2A}AR-expressing HUVECs. However, preliminary experiments suggested that in the presence of TNFα, exposure to different concentrations of forskolin demonstrated that cAMP elevation exerted different effects depending upon the concentration used. Low levels of cAMP inhibited COX-2 induction by inhibiting NFκB while high cAMP levels overcame this increasing COX-2 induction. These data suggests that the effects of A_{2A}AR expression with or without agonist on TNFα-mediated COX-2 induction can be accounted for completely by corresponding changes in cAMP. Work undertaken to identify if this cAMP-mediated increase in COX-2 induction was via PKA or EPAC pathways proved to be unsuccessful. Inhibitor experiments utilising PKA inhibitor, H89 and EPAC activator, 8- (4- Chlorophenylthio)- 2'- O-methyladenosine- 3', 5'- cyclic monophosphate (8-pCPT-2'-O-Me-cAMP; (Kraemer et al., 2001) proved inconclusive as treatment of HUVECs with H89

alone caused COX-2 induction whereas treatment with 8-pCPT-2'-O-Me-cAMP failed to induce COX-2 (results not shown).

Comparative analysis with E-selectin revealed that induction of this gene was more straightforward, being sensitive to NFκB inhibition alone with no contribution of any other signalling pathway being detectable. This is in contrast to previous studies which have shown that the cAMP response element/activating transcription factor element CRE/ATF-like domain within the E-selectin promoter (Figure 3.29) can be occupied by a variety of members of the ATF family of transcription factors, including ATF-2 ATF-a ATF-3 and c-jun (De Luca et al., 1994; Kaszubska et al., 1993). Although the nature of ATF family members bound to this element in TNFα-activated endothelial cells is uncertain, ATF-2 appears to be essential for E-selectin induction in that ATF-2 homozygous null mice are defective in E-selectin induction (Reimold et al., 1996). The activation of ATF-2 occurs by phosphorylation via activation of the JNK and p38 MAP kinases. Although both the NFκB and p38 MAP kinase pathways are activated simultaneously, there are notable differences in the duration of activation. Nuclear translocation of NFκB in endothelial cells treated with TNFα occurs by 15min and persists over many hours when TNFα is continuously present (Read et al., 1997). E-selectin transcription requires continuous presence of the activating cytokine and the continuous presence of NFκB in the nucleus (van Dam et al., 1993; Whelan et al., 1991). Although activation of JNK/p38 MAP kinases and the subsequent phosphorylation of ATF-2 and c-jun also occur within 15 minutes of TNFα stimulation, in contrast to NFκB, both JNK and p38 kinase activity and phosphorylation are transient events, (Read et al., 1997) suggesting that once transcription is initiated, sustained phosphorylation of ATF-2 and c-JUN may no longer be required which could explain why we did not observe a decrease in E-selectin induction when the cells were pre-treated with the p38 MAP kinase inhibitor (Figure 3.7).

Figure 3.29 Schematic of *E-selectin* promoter

Regulatory elements in the human E-selectin promoter indicating points of interaction by p38 MAPK inhibitor, SB203580 and NFκB inhibitor PDTC.



A_{2A}AR gene transfer suppressed TNFα-mediated E-selectin induction, but not significantly more than was observed in control cells. In contrast to the data observed with COX-2 induction, combined treatment with TNFα and CGS21680 in A_{2A}AR-expressing HUVECs showed a further reduction in E-selectin induction when compared to TNFα alone. However this was not a significant decrease when compared to control cells treated with TNFα and CGS21680. Also elevating cAMP levels in control cells showed a significant reduction of TNFα-mediated E-selectin induction when pre-treated with forskolin. The reduction in E-selectin induction in response to TNFα and cAMP elevating agents is consistent with findings by Ghersa et al. (1994) where they reported that increasing cAMP via forskolin treatment inhibited E-selectin induction.

Although it has been shown that VCAM-1 induction is solely mediated by NFκB with the VCAM-1 promoter containing two κB binding sites (Iademarco et al., 1992), our results showed that TNFα-mediated induction of VCAM-1 in HUVECs was unaffected by either A_{2A}AR gene transfer or pre-treatment of control cells with cAMP-elevating agents. This is in contrast with other reported findings where VCAM-1 is inhibited by increasing levels of intracellular cAMP although this was a IL-1-mediated effect (Ghersa et al., 1994). It can be concluded from Figure 3.7, 3.19 and 3.20 that the inhibitors and cAMP elevating agents were effective. Possible explanations could be that NFκB-regulated VCAM-1 induction occurs at an earlier time-point as observed with TNFα-mediated COX-2 induction, or VCAM-1 induction may be regulated by more than one signalling pathway and inhibition of one is not sufficient to suppress induction.

Together these data argue that the ability of A_{2A}AR to modulate expression patterns of defined κB-sensitive genes in HUVECs is dependent on the promoter of those genes. Transcription of genes which are predominantly κB-dependent such as E-selectin, is inhibited in A_{2A}AR-expressing cells and

increasing cAMP levels by stimulating adenylyl cyclase further inhibits induction of these proteins. On COX-2 where transcription is controlled by NFκB, NF-IL6 and CRE binding sites, the effect of the A_{2A}AR is far more complex. Our results indicate that at basal or slightly increased levels of intracellular cAMP the A_{2A}AR inhibits COX-2 induction by inhibition of NFκB nuclear translocation, whereas at increased levels of intracellular cAMP this is counteracted by the cAMP-dependent activation of CREB, causing a significant increase in COX-2 induction compared to control cells. The negative effect of the A_{2A}AR increasing cAMP is also consistent with the decrease in E-selectin induction, which is soley a κB-regulated event in our hands.

Examination of A_{2A}AR gene transfer on TNFα-mediated prostaglandin E_2 generation revealed that in treatment of A_{2A}AR-expressing cells with TNFα and CGS21680 resulted in a significant potentiation of prostaglandin E_2 generation when compared to control cells. These findings are consistent with the observed potentiation of COX-2 induction following the same treatment. However in contrast to the trend observed with COX-2 induction combined treatment with TNFα and CGS21680 in control cells resulted in a potentiation rather than a reduction of prostaglandin E_2 generation. This trend was also observed following identical examination of 6-keto-prostaglandin $F_{1\alpha}$. These data suggest that an inhibition of COX-2 following treatment with TNFα and an A_{2A}AR agonist does not necessarily equate to a reduction in the downstream prostanoids. Increasing cAMP concentration by treatment with rolipram and forskolin over a 24hr time-course although increasing generation of prostaglandin E_2 or 6-keto-prostaglandin $F_{1\alpha}$ by around 50% did not result in a significant increase. However it is important to note that although each sample as well as each experiment was run in triplicate, all the samples were analysed on the same ELISA plate. Therefore further analysis would need to be undertaken.

Analysis of COX-2 mRNA by quantitative PCR proved to be inconclusive. There were some problems optimising and obtaining consistent triplicates of samples which were unable to be rectified because of time and financial constraints. These problems were not due to primer derived problems as it was shown that the primers utilised produced one PCR-product from cDNA only. Therefore further work will need to be undertaken to clarify these preliminary findings.

Chapter 4

Suppression of NFκB nuclear import in vascular endothelial cells following A_{2A}AR gene transfer.

4.1 Introduction

The A_{2A} adenosine receptor ($A_{2A}AR$) is a critical non-redundant suppressor of inflammatory responses *in vivo*. While the receptor is known to elevate intracellular cyclic AMP (cAMP) levels, the molecular mechanisms underlying its inhibitory effect on defined inflammatory signalling pathways remain obscure.

The results in Chapter 3 suggest that $A_{2A}AR$ expression can inhibit the activation of the nuclear factor κB (NFκB). As discussed in detail in sections 1.4 and 1.5, NFκB is held in an inactive state in the cytoplasm of unstimulated cells by one or more members of the IκB family of inhibitor proteins which serve to mask the NLSs present on the p50 and p65 subunits (Ghosh and Karin, 2002). The degradation of IκB following targeted phosphorylation and poly-ubiquitination exposes the NLSs of p50 and p65 subunits of NFκB. The dimers are then able to translocate to the nucleus where they activate NFκB-responsive genes following binding to specific κB response elements and recruitment of transcriptional co-activatiors (Li and Verma, 2002; Li and Stark, 2002).

Eukaryotic cells are compartmentalised by the nuclear envelope into the cytoplasm and the nucleus. As discussed in section 1.5 the nuclear envelope contains nuclear pore complexes (NPCs), which mediate the molecular traffic between the two compartments. The nuclecytoplasmic traffic of large molecules (>25nm in diameter) is regulated by specific nuclear import and export systems. Proteins that contain classical NLSs are imported into the nucleus by importin α/β heterodimers. Importin-α binds to NLS containing proteins, and importin-β is responsible for the docking of the importin cargo complex to the cytoplasmic side of the NPC followed by translocation of the complex through the NPC. A classical NLS consists of a stretch of basic amino acids, arginines and lysines (Dingwall and Laskey, 1991). NFκB subunits p50

and p65 have been identified as containing classical NLSs (Blank et al., 1991; Gilmore and Temin, 1988). All importin-α molecules contain a large central motif that consists of 10 tandemly repeated armadillo (ARM) motifs. These ARM motifs mediate the interactions with the NLS-containing cargo protein as ARM repeats 2-4 comprise the N-terminal NLS binding site and ARM repeats 7-9 the C-terminal NLS binding site (Conti et al., 1998; Fontes et al., 2000; Melen et al., 2003). Of the six α importin family members identified in humans, importin-α3 and -α4 preferentially transport TNFα-activated NFκB (Fagerlund et al., 2005).

As discussed in section 1.9.2.2, the A_{2A}AR has been shown to be constitutively active. Preliminary experiments treating TNFα-stimulated A_{2A}AR-expressing HUVECs with adenosine deaminate showed no significant difference in A_{2A}AR signalling, therefore the cells are not generating lots of adenosine. Although the global classic AR antagonist ZM241385 is able to prevent agonist binding but is not an effective A_{2A}AR inverse agonist. As there is yet no effective inverse agonist for the A_{2A}AR currently available, the experiments dicussed in this chapter were undertaken examining the signalling of the A_{2A}AR in unstimulated cells.

It has previously been demonstrated in HUVECs that A_{2A}AR gene transfer causes a decrease in the nuclear accumulation of p65 (Sands et al., 2004). To identify at what point in the NFκB signalling pathway the A_{2A}AR is exerting its inhibitory effect in endothelial cells the following experiments were undertaken.

4.2 Results

Previous work carried out by our lab indicated that A_{2A}AR gene expression in HUVECs and C6 glioma cells caused a decrease in nuclear p65 accumulation in the presence of pro-inflammatory stimuli such as tumour necrosis factor-α (TNFα) and lipopolysaccharide (LPS). Confocal experiments in HUVECs suggest that A_{2A}AR-expressing cells inhibit the TNFα-mediated nuclear translocation of p65 (Sands et al., 2004). However one of the limitations of confocal experiments is that only a small number of cells can be examined, therefore to determine whether the inhibition of nuclear p65 accumulation is significant, subcellular fractionation experiments were undertaken. A_{2A}AR-expressing HUVECS were stimulated with 10ng/ml TNFα for various time-points and the cytosolic and nuclear fractions separated as described in section 2.4.5 of Materials and Methods. Nuclear fractions were then equalised for protein concentration and subjected to SDS-PAGE and immunoblotting with anti-p65 antibody and anti-SP1 antibody (a predominately nuclear protein which acts as a loading control). It can be seen in Figure 4.1 panel A that A_{2A}AR gene transfer causes a decrease in nuclear p65 at all the time-points examined compared to control cells. Panel B shows that in control cells there is a significant increase in nuclear p65 accumulation following treatment with TNFα for 15min. This is further increased at 30min, but following 60min stimulation with TNFα there is a decrease in nuclear p65 which could be explained by the resynthesis of IκBα enabling NFκB to shuttle back out of the nucleus into the cytoplasm (Verma et al., 1995). In A_{2A}AR-expressing cells the trend is similar but the level of p65 accumulation is severely inhibited over the entire time-course. As the quantity of SP1 detected remains constant in both control and A_{2A}AR-expressing cells it can be concluded that the inhibition of p65 accumulation in A_{2A}AR-expressing cells is not due to an overall decrease in nuclear protein concentration.

Figure 4.1 Effect of A_{2A}AR gene transfer on nuclear accumulation of p65 by TNFα

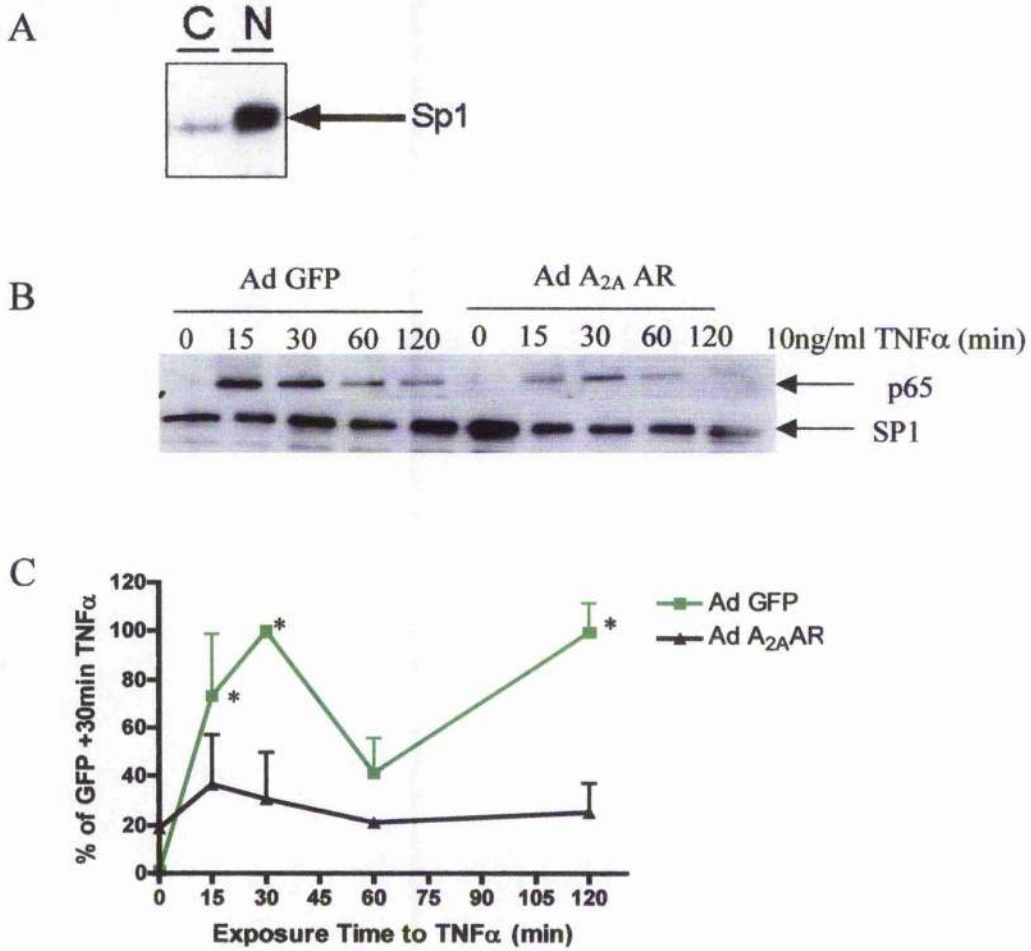
$8x10^5$ HUVECs/ dish were seeded into 12 x 10cm dishes. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad *myc*-His A_{2A}AR or Ad GFP. 48hrs post infection, cells were treated with 10ng/ml TNFα for the times shown. Cells were harvested, lysed and nuclear and cytosolic fractions separated as described in section 2.4.5. The nuclear fraction was then equalised for protein concentration and assessed for p65 and SP1 expression by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A shows the separation of nuclear (N) and cytosolic (C) fractions

Panel B represents one of 3 experiments

Panel C shows a line graph of 3 experiments for the amount of nuclear p65

**=P<0.01, *=P<0.05 when compared to vehicle



To identify whether this decrease in nuclear p65 accumulation by A_{2A}AR is due to newly synthesised IκB binding the activated NFκB and translocating the complex back out to the cytosol, A_{2A}AR-expressing cells were treated with TNFα over a 2hr time-period. Whole cell lysates were then subjected to SDS-PAGE and immunoblotting using anti-IκBα or anti-IκBε antibodies. As can be seen from the representative blot and cumulative line graph in Figure 4.2, IκBα is present in control GFP-expressing and A_{2A}AR-expressing cells prior to TNFα stimulation. After 15 min stimulation with TNFα, IκBα is no longer detectable although evidence of IκBα resynthesis is observed after 60min stimulation with TNFα. There was no significant difference observed between GFP- and A_{2A}AR expressing cells. A similar trend was observed with TNFα-mediated degradation of IκBε (Figure 4.3), however as IκBε is not a κB regulated gene there is no evidence of IκBε resynthesis. These data indicates that the A_{2A}AR inhibition of nuclear p65 accumulation cannot be explained by reduced phosphorylation-mediated degradation of IκB proteins. Therefore the effect of the A_{2A}AR must occur downstream of this step.

This leaves two possibilities for the decrease in nuclear p65 accumulation, either due to a reduced importin-mediated nuclear import of p50/p65 and/or an acceleration of CRM1-driven export of p50/p65-IκBα complexes from the nucleus back into the cytoplasm.

To discriminate between these two possibilities the *Streptomyces* metabolite Leptomycin B (LMB; Nishi et al., 1994) was utilised. LMB covalently binds to the Cys528 residue on CRM-1 preventing it from binding to the NES of IκBα in the NFκB/IκB complex (Huang et al., 2000). Initially, to ensure that the concentration of LMB was sufficient to prevent nuclear export, uninfected HUVECs was treated with LMB for 24hrs and stained for MEK1/2. MEK contains a NES which ensures that it is predominately found within the cytosol (Fukuda et al., 1996). However, as shown in Figure 4.4, 10ng/ml LMB is

Figure 4.2 Effect of $A_{2A}AR$ gene transfer on IκBα degradation by TNFα

$4x10^5$ HUVECs/well were seeded into a 6 well plate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad *myc*-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells were treated with 10ng/ml TNFα at the time points indicated. Cells were harvested, lysed and equalised for protein concentration and assessed for IκB α, and p65 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A is representative of one experiment

Panel B shows the combined results of 3 experiments for the production of IkBα ***= P<0.001, **= P<0.01, *= P<0.05 when compared with the 0 time-point. However there is no significant difference between control and $A_{2A}AR$ expressing cells.

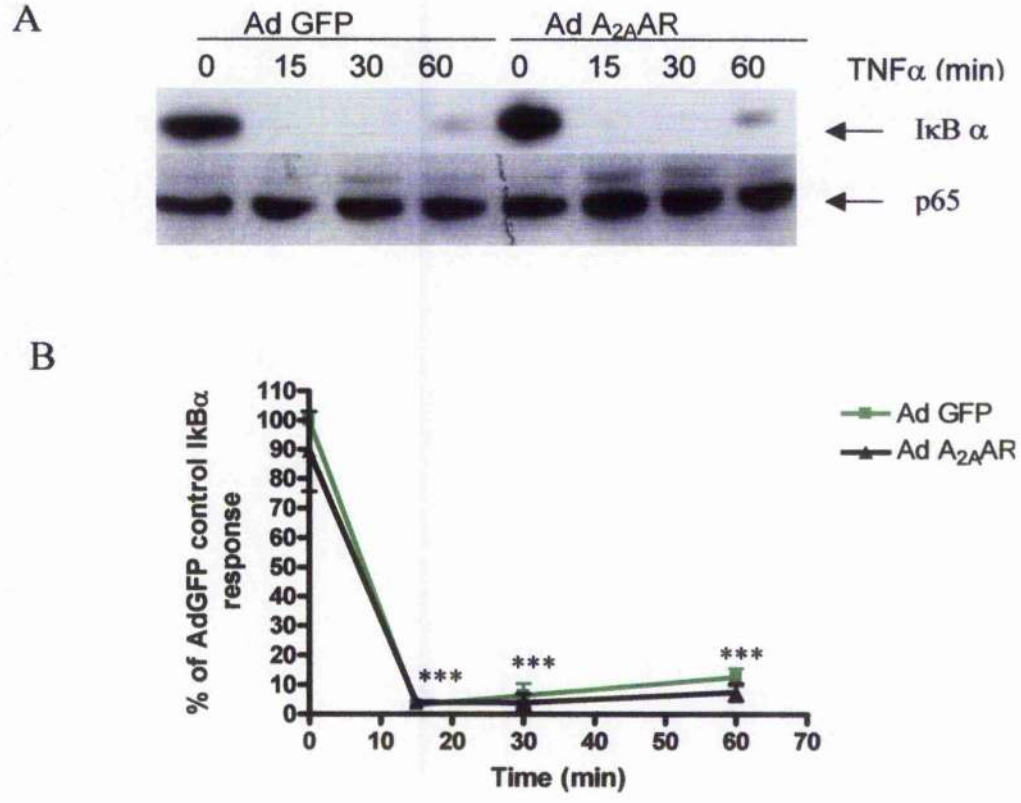


Figure 4.3 Effect of $A_{2A}AR$ gene transfer on IκBε degradation by TNFα

$4x10^5$ HUVECs/well were seeded into a 6 well plate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad *myc*-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells were treated with 10ng/ml TNFα at the time points indicated. Cells were harvested, lysed and equalised for protein concentration and assessed for IκB ε, and p65 induction by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A is representative of one experiment

Panel B shows the combined results of 3 experiments for the production of IkBε ***= P<0.001, **= P<0.01, *= P<0.05 when compared with the 0 time-point. However there is no significant difference between control and $A_{2A}AR$ expressing cells.

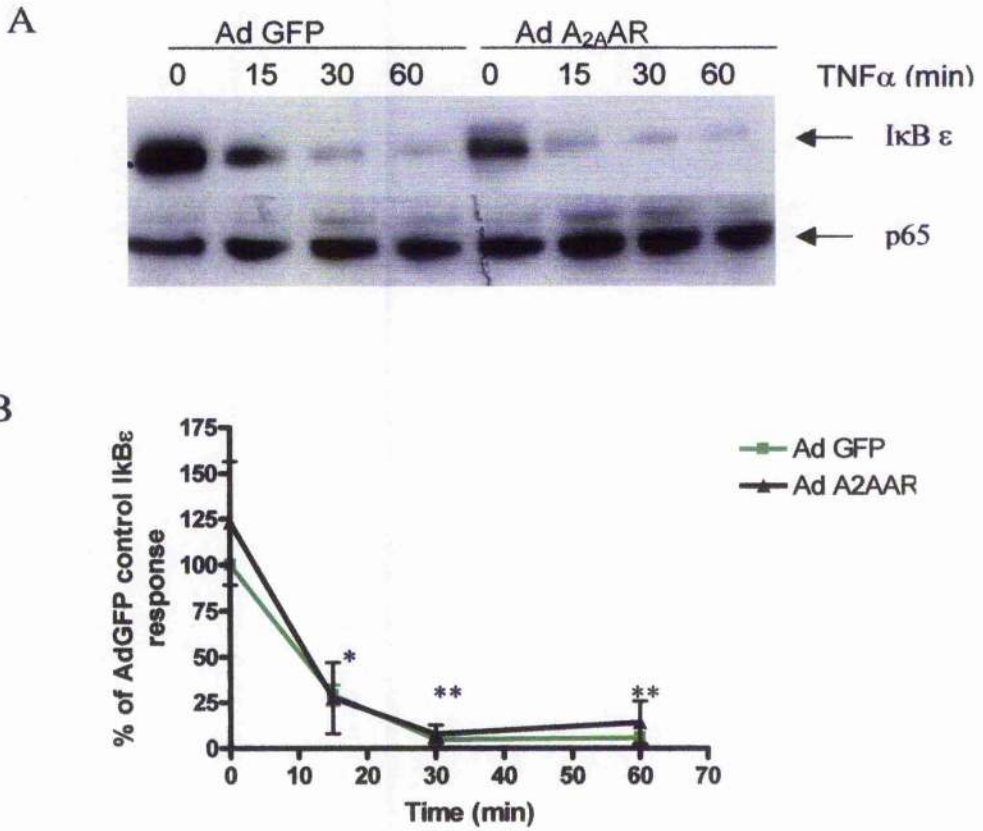
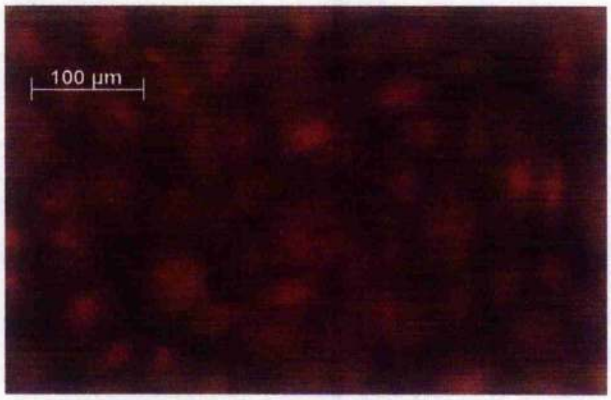


Figure 4.4 Effect of LMB on the subcellular compartmentalisation of MEK1/2 in HUVECs

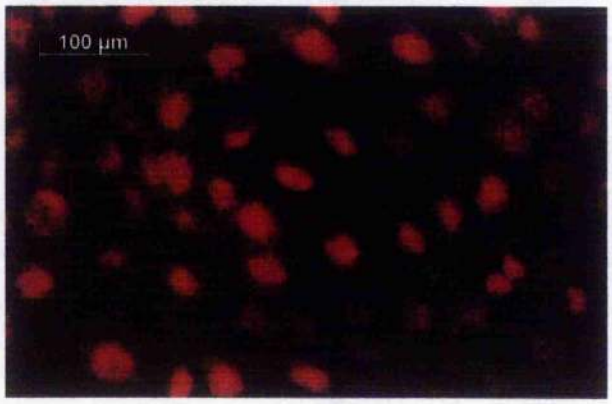
$4x10^5$HUVECs were seeded out onto poly-L-Lysine-coated cover slips in a 6 well plate. 24hrs after seeding the cells were treated with 10ng/ml LMB for 30 mins. Following treatment the cells were washed and fixed in paraformaldehyde. Cells were then semi-permeabilised and incubated with anti-MEK1/2 antibody (1:200 dilution) for 1 hour at room temperature. Cells were then rinsed and incubated with the Alexa 594-conjugated secondary antibody (1:400 dilution) for 1hr at room temperature. Following several rinses, coverslips were mounted onto microscope slides and analysed using a fluorescence microscope as indicated in section 2.4.7. Images were taken at 40X magnification.

Pictures are representative of 4 experiments

vehicle



10ng/ml LMB



sufficient to block CRM1-mediated nuclear export of MEK1, sequestering it within the nucleus. If A_{2A}AR gene transfer accelerated CRM-1-mediated export, it would be expected that pre-treatment with LMB would cause an accumulation of the NFκB/IκB complex within the nucleus. Control GFP-expressing and A_{2A}AR-expressing cells were pre-treated ± LMB and ± TNFα. In Figure 4.5, it can be seen that LMB did not have any significant effect on the inhibitory effect of A_{2A}AR gene transfer on TNFα-stimulated nuclear p65 accumulation. Thus, suggesting that the A_{2A}AR exerts its inhibitory effect via blocking nuclear import rather than accelerating export.

As mentioned earlier, IκBα is a κB target gene (Sun et al., 1993) and as such plays a role in turning off the κB response. It is believed that newly synthesised IκBα enters the nucleus where it binds to the activated NFκB and the NES on IκBα enables the complex to be translocated back into the cytosol. To determine whether the inhibition of the nuclear accumulation of p65 in A_{2A}AR-expressing cells does not involve the interaction of a κB target gene, control GFP-expressing and A_{2A}AR-expressing cells were pre-treated with a concentration of emetine (a protein synthesis inhibitor) sufficient to block IκBα resynthesis (Figure 4.6) which is required for p50/p65 nuclear export. Figure 4.7 shows that pre-treatment with emetine also failed to attenuate the inhibitory effects of A_{2A}AR gene transfer on the nuclear accumulation of NFκB as determined by EMSA analysis to assess κB binding to target DNA.

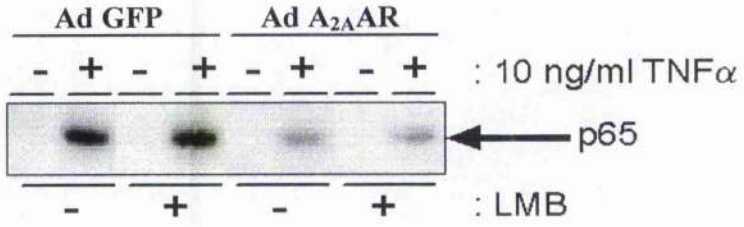
Many proteins contain cNLS, so it is important to determine whether A_{2A}AR gene transfer effects the nuclear import of all proteins which utilise the importin transport system or if it is specific to NFκB translocation. Activating transcription factor (ATF) is found predominately within the nucleus and is activated upon phosphorylation by phosphorylated p38. Inactive p38 is found in the cytosol where activation by upstream mediators causes it to be phosphorylated enabling it to be translocated to the nucleus via the importin

Figure 4.5 Effect of $A_{2A}AR$ gene transfer on TNFα-stimulated nuclear accumulation of p65 following pre-treatment with LMB

$8x10^5$HUVECs/ dish were seeded into 8 x 10cm dishes. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad/*myc*-His $A_{2A}AR$ or Ad GFP. 48hr post infection, cells were pre- treated with 10ng/ml LMB for 30 minutes and then treated ± 10ng/ml TNFα for 15 min. Cells were harvested in PBS and the nuclear and cytosolic fractions separated. Nuclear fractions were equalised for protein concentration and assessed for p65 accumulation by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A is a representative blot of three experiments.Panel B shows a line graph of the cumulative results of 3 experiments.

A



B

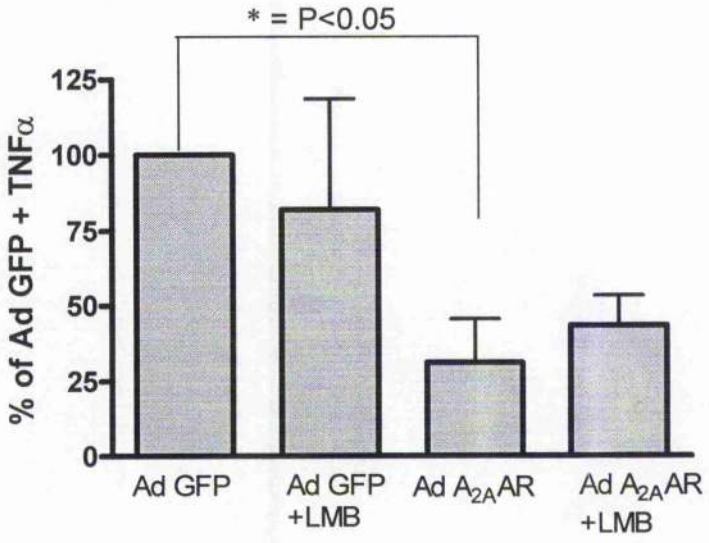


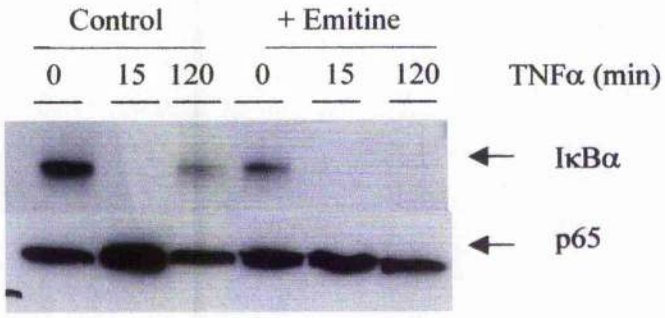
Figure 4.6 Effect of Emitine on IκBα resynthesis following TNFα stimulation

$4x10^5$ HUVECs were seeded into a 6 well plate. 24hrs after seeding out cells were pre-treated with 100μM emitine for 30 min, then treated with 10ng/ml TNFα for the times shown. Cells were harvested, lysed, equalised for protein concentration and assessed for IκBα and p65 by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A shows a representative blot of 3 experiments.

Panel B shows a line graph of the cumulative results of 3 experiments

A



B

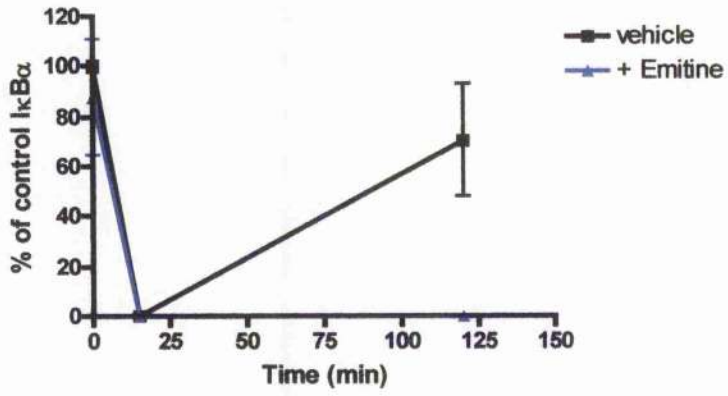
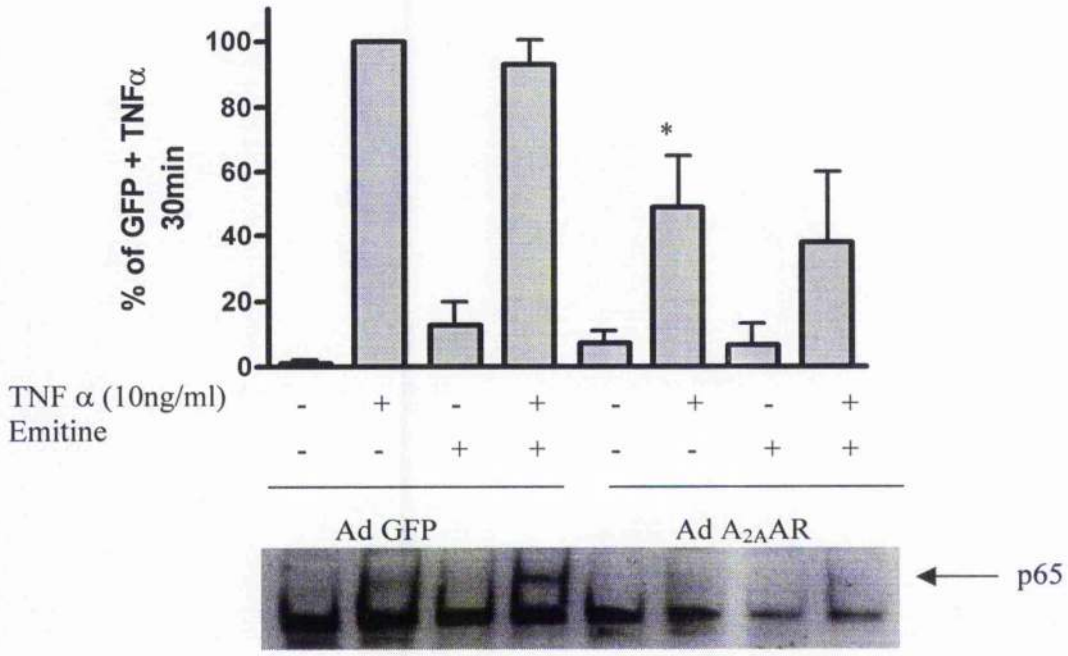


Figure 4.7 Effect of A_{2A}AR gene transfer on TNFα-mediated nuclear accumulation of p65 following pre-treatment with emitine

$8x10^5$ HUVECs/ dish were seeded into 8 x 10cm dishes. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad/*myc*-His A_{2A}AR or Ad GFP. 48hrs post infection, cells were pre-treated ± 10μM emitine for 30mins and then treated with 10ng/ml TNFα for a further 30 mins. Cells were then harvested in PBS and the cytosolic and nuclear fractions separated as described in section 2.4.5 of Materials and Methods. Protein concentrations of nuclear fractions were determined and equal protein concentrations of samples were analysed by EMSA as described in section 2.4.6 of Materials and Methods.

*= P<0.05 there is a significant reduction in p65 in A_{2A}AR-expressing cells compared to the control, but there is no significant difference between cells treated with emitine and TNFα and those only treated with TNFα.



transport system where it is able to phosphorylate ATF (Read et al., 1997). Examination of TNFα-stimulated control GFP-expressing and A_{2A}AR-expressing HUVECs by SDS-PAGE and immunoblotting with anti-pATF, anti-p38 and anti-total p38 antibodies, identified that there was no significant difference between control GFP-expressing and A_{2A}AR-expressing HUVECs. A_{2A}AR gene transfer did not prevent phosphorylated p38 translocation into the nucleus enabling it to phosphorylate the nuclear protein, ATF (Figure 4.8). Therefore, suggesting that the inhibition of nuclear p65 accumulation by A_{2A}AR gene transfer is specific to p65 translocation and not a global effect of all nuclear imported proteins.

Work undertaken by Fagerlund et al., (2005) showed that the classical NLS on both p50 and p65 NFκB subunits, preferentially binds to importin-α3. To determine if A_{2A}AR gene transfer inhibited p65/importin-α3 binding the following experiments were undertaken. 5μg of GST- and GST-importin-α3-immobilised Sepharose beads were added to control cells following treatment with TNFα over a 1hr time-period. Figure 4.9 shows that there was no interaction between GST-importin-α3 and p65 prior to TNFα treatment. Following 15min of TNFα treatment, there is a significant interaction between p65 and GST-importin-α3 which decreases overtime but still shows significant binding. The interaction is specific since it was shown to occur between importin-α3 and not GST alone as p65 was not detected within these samples. The use of Ponceau S stain shows that equal quantities of protein was loaded onto the gel. When examined in A_{2A}AR-expressing HUVECs, it was observed that at this concentration of GST-importin-α3 there was no significant effect on the ability of importin-α3/p65 to bind when compared to control GFP-expressing cells (Figure 4.10).

4.3 Discussion

It has been reported in several cell types, including HeLa and cultured endothelial cells, adenosine-mediated inhibition of NFκB can be observed

Figure 4.8 Effect of $A_{2A}AR$ gene transfer on phosphorylated p38 and phosphorylated ATF-2

$4x10^5$ HUVECs were seeded into a 6 well plate. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad/*myc*-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells were treated with 10ng/ml TNFα for the times indicated. Cells were harvested, lysed, equalised for protein concentration and assessed for phospho-ATF-2, phospho-p38 and total p38 levels by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A is a representative blott of three experiments

Panel B is a line graph of phospho-ATF-2 expression

Panel C is a line graph of phospho-p38 expression

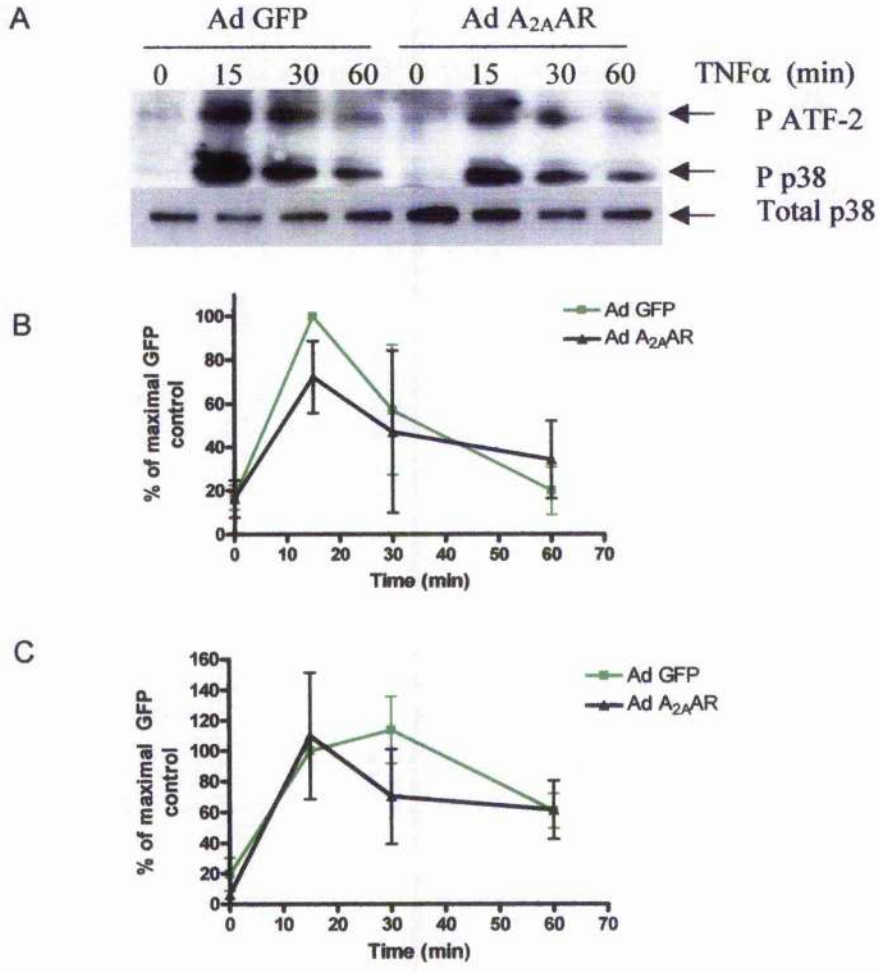


Figure 4.9 Effect of TNFα on p65-GST- importin α-3 interaction *in vitro*

$8x10^5$ HUVECs/ dish were seeded into 8 x 10cm dishes. 24hrs after seeding, cells were treated with 10ng/ml TNFα for the times indicated. Cells were then harvested, lysed, equalised for protein concentration and incubated with 5μg GST-importin α-3-imobilised or GST-immobilised Sepharose beads for 2hrs at 4°C as described in section 2.4.9. The beads were then washed and bound protein eluted in 12% SDS sample buffer for 15 mins at 65 °C. Eluate and cell lysates were assessed for p65 content by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A shows representative blots from one experiment

Panel B shows a line graph of cumulative results of 3 experiments for p65 bound to GST-importin α-3 beads

**= P<0.1 and *= P<0.5

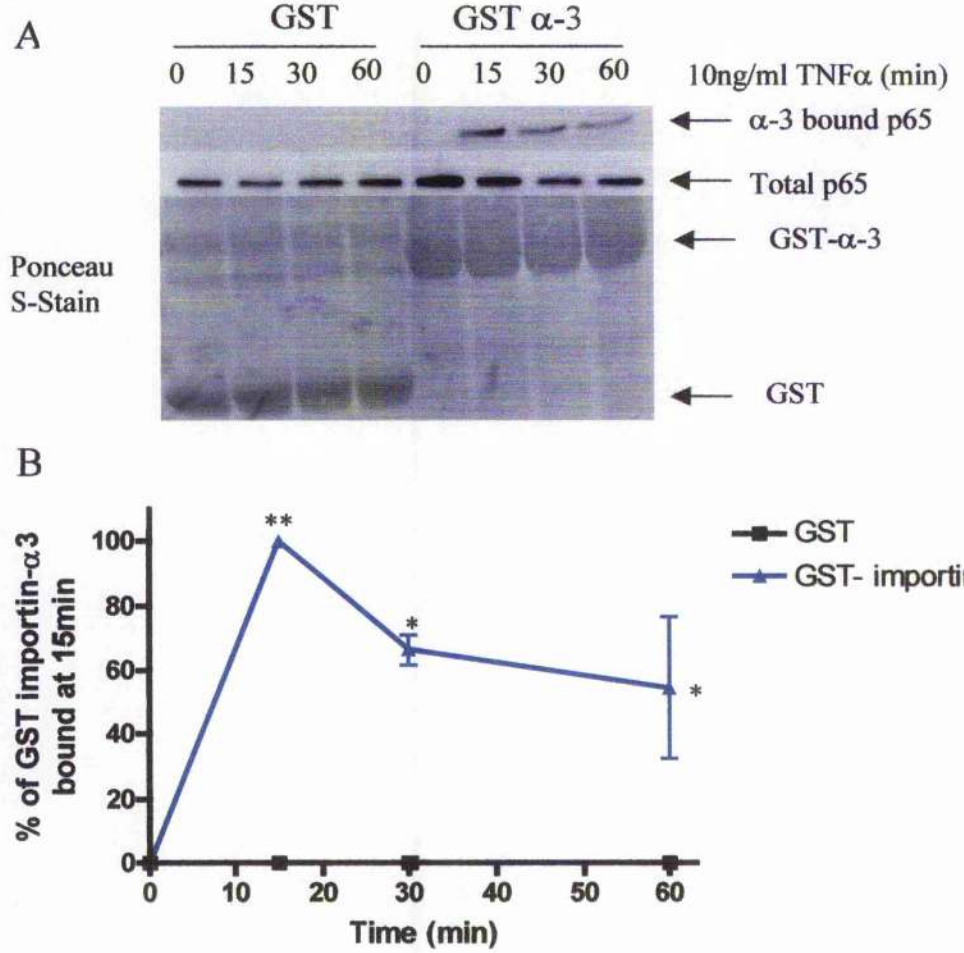
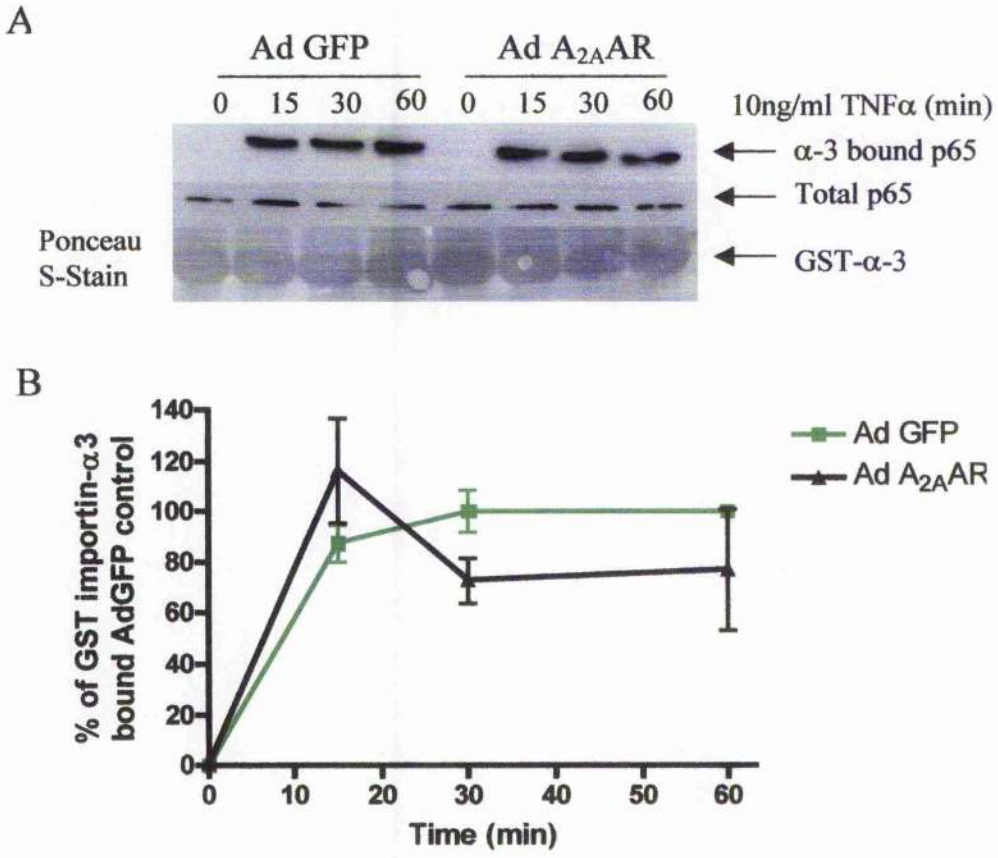


Figure 4.10 Effect of $A_{2A}AR$ gene transfer on importin α-3 /p65 interaction *in vitro*

$8x10^5$HUVECs/ dish were seeded into 8 x 10cm dish. 24hrs after seeding, cells were infected with 125pfu/cell of recombinant Ad/*myc*-His $A_{2A}AR$ or Ad GFP. 48hrs post infection, cells were treated with 10ng/ml TNFα for the times indicated. Cells were harvested, equalised for protein concentration and incubated with 5μg GST-importin α-3 beads for 2hrs at 4°C as described in section 2.4.9. The beads were then washed and bound protein eluted in 12% SDS sample buffer for 15 mins at 65 °C. Eluate and cell lysates were assessed for p65 content by SDS-PAGE and immunoblotting as described in section 2.4.3.

Panel A shows representative blots from one experiment

Panel B shows a line graph of the cumulative results of 3 experiments for p65 bound to GST-importin α-3 beads



(Majumdar and Aggarwal, 2003). Pharmacological analysis ruled out the contribution of A_1 and A_3ARs, but did not address the specific involvement of A_{2A} or A_{2B}ARs in each of the cell types under investigation. Work presented by Sands et al., (2004) indicated that it was the A_{2A}AR subtype which was largely responsible for the inhibition of NFκB. Majumdar and Aggarwal (2003) also noted that adenosine specifically inhibited TNFα-induced NFκB activation and nuclear translocation in the absence of any effect on IκB degradation.

Our results demonstrate that in A_{2A}AR-expressing HUVECs there is an inhibition of NFκB activation and nuclear translocation. Consistent with the findings of Majumdar and Aggarwal (2003) this effect appears to occur downstream of IκB degradation. Therefore the effect of the A_{2A}AR inhibiting NFκB nuclear accumulation must be due to either reduced nuclear import of p50/p65 and/or accelerated CRM-1-mediated export of IκBα/NFκB complexes out of the nucleus (Arenzana-Seisdedos et al., 1997).

However, as our results showed that there was not a significant increase in TNFα-mediated nuclear p65 in LMB pre-treated A_{2A}AR-expressing cells when compared to those not treated with LMB. This suggests that A_{2A}AR gene transfer reduced nuclear import by an as yet undetermined method. Consistent with these findings were our observations that pre-treatment with the protein synthesis inhibitor emitine, at a concentration which inhibited IκBα resynthesis in control cells, did not significantly increase the TNFα-mediated nuclear p65 in A_{2A}AR-expressing cells. However it should be noted that there were some difficulties using the EMSA method described in section 2.4.6 and it would be prudent to repeat this experiment using a hot EMSA method.

p38 is a predominately cytosolic protein, when phosophorylated it translocates to the nucleus where it subsequently phosophorylates the nuclear protein ATF (Read et al., 1997). Our results indicate that the effect of A_{2A}AR gene transfer on nuclear p65 accumulation specific to p65 as there was no observed

decrease in phosphorylated p38 and phosphorylated ATF in A_{2A}AR expressing cells when compared to control cells, ruling out a global effect.

NFκB translocates to the nucleus via the nuclear pore complex (NPC) in a complex with the importin α/β heterodimer as discussed in Section 1.8. It has been shown that the NLS's on both p65 and p50 preferentially bind to importin-α3 (Fagerlund et al., 2005). Our results show that p65 does indeed bind to importin-α3, however there is no difference in binding between A_{2A}AR-expressing cells and control cells. This was unexpected as it was hypothesised from our other observations that the A_{2A}AR was inhibiting nuclear import of p65 via the importin-α3.

Importin-α subunits contain an importin-β binding (IBB) domain at its N-terminal which serves a dual role. It binds to importin-B to target the complex to the NPC for nuclear translocation (Gorlich et al., 1996; Weis et al., 1996) but it also contains an auto-inhibitory sequence that mimics a classical NLS (cNLS) such as those on p65 and p50. This regulates binding of cNLS cargo to the ARM domain of importin-α. (Fanara et al., 2000; Morolanu et al., 1996) The auto-inhibitory sequence apparently interacts with the NLS-binding pocket when importin-α is not bound to importin-β. (Kobe, 1999) This interaction is not exceptionally strong as cNLS cargos can still bind to importin-α in the absence of importin-β although at a lower affinity. The order of binding of importin-α to its cNLS cargo and importin-β is not full understood and is suggested that it varies depending on the particular cNLS cargo (Goldfarb et al., 2004). Either way the relationship of importin-α/β and its cNLS cargo has to be bipolar, forming tight complexes in the cytoplasm then switching to an extremely low affinity state in the nucleus to release the cNLS cargo.

If the A_{2A}AR is not inhibiting the binding of p65 to importin-α3 there are other areas in the nuclear import pathway that the A_{2A}AR could be effecting. One

possibility is that the $A_{2A}AR$ could be preventing the binding of p65/importin-α3 to importin-β therefore preventing the complex from being targeted for nuclear translocation. This could be examined by using Sepharose bound GST-importin-β, as this should pull-down the importin-α3 complex. Integrating p65 into co-immuno-precipitation experiments would also be useful in identifying whether importin-α3/β complexes could be pulled-down in $A_{2A}AR$-expressing cells. However as the order of binding is not fully understood if importin-α binds to importin-β before to the cNLS, further experiments would need to be undertaken.

Due to the nature of the pull-down experiments importin-α3 is present in excess and the $A_{2A}AR$ may be inhibiting binding to the importin-α subunit but this inhibition could not be observed. To clarify this, further pull-down experiments would need to be undertaken utilising decreasing concentrations of the GST-importin-α3 bound Sepharose beads. Western blotting could also be utilised to determine if there is a decrease in free importin-α in the cytoplasm as it has been reported that CAS, the export receptor for importin-α (Katay et al., 1997), when in excess, could lead to constitutive binding with importin-α and RanGTP, depleting cytoplasmic stores of free importin-α and thus inhibiting nuclear import (Kau et al., 2004). CAS is phosphorylated by MEK1 and treatment with MEK1 inhibitors can alter the nuclear/cytoplasmic distribution of CAS (Scherf et al., 1998).

Another possibility is that the $A_{2A}AR$ causes a post-translational modification of the activated NFκB, such as site-specific phosphorylation, acetylation or ubiquitination. It has been reported that to achieve a maximal transcriptional response, the p65 subunit of NFκB must undergo post-translational modifications including phosphorylation (Chen and Greene, 2004). One of the key phosphorylation events involves that action of the catalytic subunit of PKA (PKA_c), which modifies Ser276 within the RHD of p65. This phosphorylation

event occurs within the cytosol and regulates the DNA-binding and oligomerisation properties of NFκB (Ganchi et al., 1993; Mosialos and Gilmore, 1993). Although the majority of phosphorylation and acetylation events exert a positive effect on the transcriptional activity of NFκB (Chen and Greene, 2004), it is possible that a post-translational modification could prevent NFκB/importin binding and subsequent translocation into the nucleus. One method that could be utilised to identify any modification would be via 2-dimentional electrophoresis gels. Any modification would result in a difference in charge and size which could be identified when the gels were compared. This method was attempted but due to time and optimisation constraints it was not possible to obtain any reproducible results. Therefore further work would need to be undertaken.

Taken together these data argue the ability of A_{2A}AR to inhibit TNFα-mediated nuclear accumulation of p65 in endothelial cells is due to a reduced ability of p50/p65 dimers to undergo importin-mediated nuclear import. However, further work is required to determine where in the importin nuclear import pathway the A_{2A}AR is exerting its effect.

Chapter 5
Final Discussion

Inappropriate or prolonged inflammation contributes to the pathogenesis of many diseases including atherosclerosis, rheumatoid arthritis, sepsis, heart disease and cancer (D'Cruz, 1998; Greaves and Channon, 2002; Gueler et al., 2004; Karouzakis et al., 2006; Kinlay et al., 2001; Zimmerman et al., 1999). Fundamental to the inflammatory response is the interaction between endothelial cells and leukocytes. These interactions trigger further downstream signalling events including cytokine, chemokine and growth factor release, surface expression of adhesion molecules and expression of other pro-inflammatory proteins (Section 1.2). It is widely accepted that adenosine is a potent inhibitor of inflammatory processes and that the A_{2A}AR plays an important role as a suppressor of inflammation (Lappas et al., 2005). Therefore understanding the mechanisms behind the suppression of inflammation would be beneficial in providing future drug targets for chronic inflammatory diseases. It has been reported that A_{2A}AR gene transfer suppresses p65 nuclear translocation and subsequently NFκB activation in response to pro-inflammatory stimuli such as TNFα and LPS (Sands et al., 2004). Results presented in this thesis have demonstrated that A_{2A}AR-expressing cells exhibit an inhibitory effect on TNFα-mediated protein induction of primarily κB-dependent target genes. However activation of the A_{2A}AR causes potentiation of protein induction on target genes which require both κB and other elements for optimal gene transcription. Additional data also shows that the inhibitory effect of the A_{2A}AR on nuclear accumulation of p65 occurs by inhibiting the nuclear import of NFκB.

In Chapter 3, it was shown that TNFα-mediated E-selectin induction was significantly inhibited in A_{2A}AR-expressing HUVECs and that activation of the A_{2A}AR resulted in the further inhibition of E-selectin induction. It has been reported that although E-selectin gene transcription requires activation of ATF-2 (Reimold et al., 1996) and NFκB, once transcription is initiated ATF-2 is no longer required (Read et al., 1997). This is consistent with our findings that the inhibition of TNFα-mediated E-selectin induction in A_{2A}AR-expressing HUVECs

was found to be solely due to the inhibition of NFκB (Sands et al., 2004). This could explain the observed A_{2A}AR activation resulting in a decrease in leukocyte-mediated inflammatory responses (Kelly et al., 2003)

VCAM-1 induction has previously been reported to be a solely κB regulated gene due to the two κB binding sites present at –77 and –63 of the VCAM gene (lademarco et al., 1992). In contrast to these findings, our data failed to show significant inhibition of TNFα-mediated VCAM-1 induction following pre-treatment with the NFκB inhibitor PDTC. In addition, neither A_{2A}AR gene transfer nor pre-treatment with elevating cAMP agents had any effect on TNFα-mediated VCAM-1 induction which is in contrast to the findings of Ghersa et al. (1994) where elevating intracellular cAMP concentrations inhibited IL-6-mediated VCAM-1 induction. Further analysis is required to completely understand the mechanisms involved in VCAM-1 induction. One possibility is that there was a loss of VCAM-1 from the cell surface during the ELISA protocol. This could be examined by western blot analysis. Other possibilities are that NFκB-regulation of VCAM-1 induction occurs at an earlier time-point than the one examined or there are other signalling pathways involved in VCAM-1 induction. Chromatin immunoprecipitation (CHIP) analysis of the VCAM-1 promoter region could provide some insight into whether other signalling pathways are involved. CHIP analysis enables the rapid identification of the precise binding sites of specific DNA binding proteins including transcription factors and histones within large streatches of target DNA.

In HUVECs, A_{2A}AR gene transfer had a dual regulation of TNFα-mediated COX-2 induction. In A_{2A}AR-expressing cells TNFα-mediated COX-2 induction was inhibited however in the presence of A_{2A}AR-selective agonist CGS21680, TNFα-mediated COX-2 induction was significantly potentiated. Further analysis utilising the cAMP elevating agents rolipram and forskolin, suggested that the effects of A_{2A}AR expression with or without agonist on TNFα-mediated COX-2 induction could be accounted for completely by corresponding changes in

cAMP. These findings are consistent with the report by Schroer et al. (2002), which indicated that elevation of cAMP by either PGI_2, PGE_2 or forskolin amplifies COX-2 promoter activity by increasing CREB binding to the CRE element, although they were unable to detect an increase in COX-2 protein levels. Preliminary attempts to identify if the cAMP-mediated increase in COX-2 induction was via PKA or EPAC pathways have proved inconclusive. This was in part due to PKA inhibitor, H89-mediated COX-2 induction in the absence of TNFα and the failure of EPAC activator, 8-pCPT-2'-O-Me-cAMP to induce COX-2. Determining the role of cAMP on the potentiation of COX-2 induction could be undertaken by utilising siRNA to knockdown CREB or identification of the proteins bound to the COX-2 promoter in A_{2A}AR-expressing cells by promoter immunoprecipitation or CHIP analysis. With regards to our data, CHIP analysis could be used to identify the transcription factor bound to the COX-2 promoter.

The up-regulation of COX-2 is associated with a number of disease states including myocardial infarction, arthritis, urogenital disease and cancer (Turini and DuBois, 2002). These data indicate that expression of the A_{2A}AR would result in constitutive downregulation of COX-2 induction which in these disease states would be beneficial. However, as it has been shown that activation of the A_{2A}AR results in a significant potentiation of COX-2 induciton, tissues that express high levels of A_{2A}AR would, if activated by a local increase in adenosine, result in an increase in COX-2 metabolites. As the A_{2A}AR is expressed within blood vessels, leukocytes, the spleen and thymus, an increase in local adenosine concentrations could have implications in disease in these tissues. The recent findings that NSAID COX-2 inhibitors have been found be associated with an increased risk of myocardial infarction in susceptible patients (Bombardier et al., 2000; Caughey et al., 2001; Mitchell and Warner, 2006) means that elucidating the mechanism of COX-2 induction would provide valuable insight into the global effects these inhibitors can cause and enable more selective drug targets to be identified.

In Chapter 4 it was reported that A_{2A}AR inhibited nuclear accumulation of p65 was due to an inhibition of nuclear import rather than accelerated nuclear export. Other groups have previously reported that the effect of the A_{2A}AR to inhibit NFκB nuclear accumulation occurs downstream of IκB degradation and must be due to either reduced nuclear import of p50/p65 and/or accelerated CRM-1-mediated export of IκBα/NFκB complexes out of the nucleus (Arenzana-Seisdedos et al., 1997; Majumdar and Aggarwal, 2003). The inability of CRM-1 treatment to rescue the A_{2A}AR-mediated inhibition of p65 nuclear accumulation ultimately means that the effect of the A_{2A}AR has to be inhibiting nuclear import. The translocation of NFκB into the nucleus requires the interaction of importin-α, importin-β/RanGTP and the NPC (Fagerlund et al., 2005; Goldfarb et al., 2004). However, A_{2A}AR-expressing cells did not show any inhibition in p65/importinα-3 interaction in GST-pulldown experiments. One possibility is that the concentration of GST-importinα-3 was in excess which could be masking any A_{2A}AR-mediated changes in p65/importin-α3 binding. To address this, pulldown experiments utilising varying concentrations of GST-importin-α3 bound Sepharose beads could be undertaken. In addition co-immunoprecipitation experiments could be undertaken to identify if A_{2A}AR-expression results in a decrease in p65/importin-α3 interaction. Another consideration is that an excess of the nuclear export protein CAS can lead to the constitutive binding of imortin-α and RanGTP resulting in a decrease in cytoplasmic stores of free importin-α (Kau et al., 2004). Western blot analysis could be utilised to identify any decreases in cytoplasmic importin-α. It is possible that A_{2A}AR does not effect p65/importin-α binding, but it may effect the interaction of p65/importin-α with importin-β and/or the NPC. Co-immunoprecipitation experiments would thus be useful to identify if there is an inhibition in importin-α/-β binding. It is important to note that the exact order of binding between the NLS-containing protein, importin-α and importin-β is not fully understood which may impede the understanding of A_{2A}AR inhibition of the nuclear import of NFκB.

It is known that NFκB is subjected to regulation by post-translational modifications such as site-specific phosphorylation, acetylation or ubiquitination (Chen et al., 2005; Greene and Chen, 2004). 2-dimensional gel electrophoresis was attempted to identify if the A_{2A}AR mediated changes in the pattern of p65 post-translational modification. Unfortunately due to time constraints it was not possible to obtain any reproducible results. Optimisation of this process could lead to the identification of a post-translational modification of NFκB in A_{2A}AR-expressing cells. There are other methods which can be utilised to identify the type of possible post-translational modification. Possible post-translational modifications include phosphorylation, ubiquitination and acetylation. As any possible modification of p65 is preventing nuclear translocation, acetylation, which occurs predominately within the nucleus, will not be discussed here.

TNFα-mediated activation of p65 requires site-specific phosphorylation prior to nuclear import (Section 1.34). There are a few methods which could be undertaken to determine whether A_{2A}AR-expression results in a change in site-specific phosphylation of p65 that inhibits nuclear import. A change in the phosphorylation pattern of p65 in A_{2A}AR-expressing cells could be identified by P^{32} metabolic labelling experiments followed by immunoprecipitation of p65 or 2-dimensional gel electrophoresis. If this is successful the sequence can be examined for consensus phosphorylation sites and site-directed mutagenesis can be undertaken to identify the role, if any, of these sites in the inhibition of NFκB nuclear import. Another possibility is that accessory proteins such as Pin-1 or 14-3-3, which are recruited to specific phosphorylated sites on p65, prevent interaction with the nuclear import machinery. Pin-1 is a proline isomerase that binds and isomerases specific phosphorylated Ser or Thr residues that precede proline in certain proteins. It has been reported that Pin-1 directly binds to the phosphoylated Thr254-Pro motif on NFκB which is exposed upon IκB degradation, causing a conformational change (Ryo et al., 2003). It is possible that the binding of a Pin-like protein which causes a conformational

change in NFκB assists the A_{2A}AR-mediated inhibition of NFκB nuclear import. To identify if phosphorylation of p65 results in changes in p65/importin-α binding, pulldown experiments utilising GST-Pin-1 could be undertaken. 14-3-3 are Ser/Thr binding proteins are established as a large family (over 100 members identified) of dimeric proteins that can modulate protein interactions required for intracellular trafficking and transcription (Aitken, 2006). In most cases the binding occurs through a consensus motif within the target protein, which contains a phosphorylated Ser or Thr residue (Muslin et al., 1996), although some cases have been reported where phosphorylation of 14-3-3 proteins themselves may modulate interaction (Aitken, 2006). It has been reported that PKB-mediated phosphorylation of Thr157 on the cyclin-dependent kinase (CDK) inhibitor p27 promotes the binding of 14-3-3. Thr157 is within the importinα-3 specific NLS binding site of p27 and the binding of 14-3-3 to this site prevents p27/importin α-3 interactions, resulting in the inhibition of nuclear import (Sekimoto et al., 2004). As p65 also binds to importin α-3, it is possible 14-3-3 could also be responsible for the A_{2A}AR-mediated inhibition of p65 nuclear import. This could be identified by GST-14-3-3 pulldown experiments, however as there are over 100 members of the 14-3-3 family it would be beneficial to identify whether 14-3-3 binds to p65 by immunoprecipitating p65 from TNFα-stimulated A_{2A}AR-expressing HUVECs and identify any phosphorylated 14-3-3 binding motifs by western blot.

There are recent reports that NFκB signalling pathways are subject to ubiquitination (Chen, 2005). Along with the traditional role of ubiquitin, targeting IκB via Lys 48 polyubiquitination for protesomal degradation (section 1.4.3), polyubiquitin chains linked by Lys 63 have recently been found to regulate DNA repair and protein-kinase activation through a degradation-independent mechanism (Chen, 2005). Our data indicates that A_{2A}AR-expressing cells do not inhibit p65 by targeting it for degradation in the proteasome through ubiquitin Lys 48 linkage, although it is possible that polyubiquitination can occur via another Lys residue. Possible methods for identification of ubiquitination

include treatment of A_{2A}AR-expressing cells with a proteosome inhibitor and then analysing p65 by immunoblotting. If p65 is polyubiquitinated then a ladder effect should be observed on the blot at 8kDa intervals. However this is not always effective as the specific antibodies may not recognise the ubiquitinated protein. Another method would be to immunoprecipitate the target protein and then probe by western blot using an anti-ubiquitin antibody. Lys 63-linked polyubiquitination is known to be associated with protein/protein interactions (Vong et al., 2005). To identify whether p65 is Lys63 polyubiquitinated or only monoubiquitinated, either L63/Arg or all Lys/Arg mutants could be generated. These could then be transfected into cells and p65 immunoprecipitated following TNFα stimulation. If p65 is Lys63 polyubiquitinated then it would not be detected in L63/Arg mutants, however if p65 is monoubiquitinated then p65 would be detected in Lys/Arg mutants.

Although this thesis has focused on the p50/p65 heterodimer of NFκB it would be interesting to see if the A_{2A}AR-mediated inhibition of NFκB nuclear import occurs with the other NFκB dimers as some combinations are known to repress transcription (p50/p50 homodimers; Zhong et al., 2002)

While it is important to analyse the precise role of A_{2A}AR regulation of NFκB activation with respect to inflammation, NFκB has also emerged as a critical regulator of tumourogenesis and cancer (Garg and Aggarwal, 2002; Perkins, 2004). This can partly be explained by its ability to regulate the expression of a number of genes which are involved in the regulation of apoptosis and tumour progression (Garg and Aggarwal, 2002). Inhibition of NFκB by A_{2A}AR activation could also be able to sensitise cells to apoptosis and promote tumour growth. Sensitisation to apoptosis would be of great benefit in treating tumours, although it could also lead to undesirable side effects such as the premature apoptosis of immune cells leading to an immunocompromised situation. However it is important to note that NFκB has been reported to actively repress anti-apoptotic gene expression when activated by UV-C and chemotherapeutic

drugs such as daunorubicin (Campbell et al., 2006). This inhibition which is mediated by the association of p65 with HDAC, therefore indicates that NFκB has the ability to act as both a transcriptional activator or suppressor dependent upon the signalling pathway of induction (Campbell et al., 2004)

In conclusion, A_{2A}AR inhibits the nuclear accumulation of NFκB by specifically inhibiting nuclear import, resulting in the inhibition of κB-regulated E-selectin induction. This is not the case for the target gene COX-2 which is regulated by both κB and CRE elements. The A_{2A}AR appears to have a dual role in the regulation of COX-2. Low concentrations of cAMP results in the inhibition of COX-2 induction whereas increasing intracellular cAMP concentrations by signalling from activated A_{2A}ARs, results in the potentiation of COX-2 induction and subsequent up-regulation of COX-2 metabolites.

Chapter 6
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