The role of purinergic receptors in the modulation of JNK signalling in endothelial and cancer cells

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Abstract (303 words)

Purinergic receptors (P2YR) are a family of G-protein coupled receptors (GPCRs) which have been shown to regulate MAPK (mitogen activated protein kinase) signalling to elicit pro-inflammatory responses in diseases such as atherosclerosis.[1,2] Within the MAPK family, JNK (c-Jun NH_2-terminal kinase) is strongly activated by pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).[3,4] Thus, this project examined the potential for purinergic and cytokine receptors to interact at the signalling level. In particular, it was hypothesised that purinoceptors could inhibit cytokine induced JNK signalling.

In human umbilical vein endothelial cells (HUVECs), activation of P2YR by ATP produced a concentration dependent inhibition of TNF-α and IL-1β mediated JNK activity, as measured by Western blotting and *in vitro* kinase assays. This inhibitory effect was cytokine specific, as neither sorbitol nor anisomycin dependent JNK activity was affected. Furthermore, ATP altered neither cytokine mediated p38 MAPK nor NF-κB signalling, indicating that the effect was pathway specific. In order to identify the P2YR involved, HUVECs were pre-treated with antagonists for $P2Y_{11}$ (NF340) or $P2Y_1$ (MRS2179), but only NF340 reversed the inhibitory effect of ATP. The signalling pathways involved were also studied using the $G_{q/11}$ inhibitor YM254890 and the PKA inhibitor H89, which produced a partial and complete reversal of ATP mediated inhibition respectively. ATP mediated JNK inhibition was also translated into a physiological outcome via a reduction in IL-1β dependent cyclo-oxygenase 2 expression.

In MDA-MB-231 breast cancer cells, ATP and adenosine also inhibited cytokine dependent JNK signaling. Surprisingly, ATP inhibited the UVC-mediated JNK signalling, which was completely reversed by the $G_{q/11}$ inhibitor YM254890. The JNK dependent apoptotic effect of UVC was also examined, but neither the JNK inhibitor SP600125, nor ATP had a significant effect on apoptosis.

In conclusion, this study supported an emerging signalling paradigm that GPCR activation can negatively modulate JNK signalling in endothelial and cancer cells.

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University of Strathclyde Research Day, Barony Hall, 7th June 2011:

Pei Yuen Ng, Kathryn McIntosh, Robin Plevin. **A strategy to combat heart disease – Signalling interaction between receptor families in endothelial cells** (Abstract ID: 48332)

Joint Cell Biology and Integrative Mammalian Biology Postgraduate Research Day, SIPBS, 12th May 2011:

Pei Yuen Ng, Kathryn McIntosh, Robin Plevin. **Purinergic receptor mediated inhibition of cytokine driven JNK signalling**

BPS winter meeting 2010, Queen Elizabeth II Conference Centre, London, 14 – 16th December 2010 (abstract published):

Pei Yuen Ng, Kathryn McIntosh, Robin Plevin. **Protective model of G-protein coupled receptor activation in reducing cytokine signalling (TNF-α and IL-1β) in HUVECs (P066)**

16th World Congress of Basic and Clinical Pharmacology 2010, Copenhagen, Denmark, 17 – 23th July 2010 (abstract published):
Robin Plevin, F Goon Goh, PY Ng. **Dual effect of antagonist K-14585 on PAR-2 pro-inflammatory signalling**. Basic Clin Pharmacol Toxicol. 2010 Jul; Suppl 1:529

Oral Presentation

Joint Cell Biology and Integrative Mammalian Biology Postgraduate Research Day, SIPBS, 6th May 2009: **GPCR modulation of cytokine responses in inflammatory conditions**: Best 1st year oral presentation for cell biology group

Publication

Goh FG, Ng PY, Nilsson M, Kanke T, Plevin R. **Dual effect of the novel peptide antagonist K-14585 on proteinase-activated receptor-2-mediated signalling**. *Br J Pharmacol.* 2009 Dec; 158(7): 1695-704

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List of abbreviations

AAA	Abdominal aortic aneurysm
AP-1	Activator protein 1
6-4 PP	6-4 photoproduct
7-AAD	7-amino-actinomycin
ADP	Adenosine diphosphate
ApoE	Apolipoprotein E
ASK1	Apoptosis signal regulating kinase-1
AT_1	Angiotensin II type1 receptor
ATP	Adenosine-5'-triphosphate
BAD	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bcl2	B-cell lymphoma 2
Bid	BH3 interacting-domain death agonist
Bim_{EL}	Bcl-2-like protein 1-extra long
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid receptor
CCK	Cholecystokinin octapeptide
cdc	cell division cycle
c-FLIP	Cellular FADD-like IL-1β-converting enzyme inhibitory protein
cIAP1/2	Cellular inhibitor of apoptosis 1/2
CPD	Cyclobutane pyrimidine dimer
CREB	cAMP responsive element binding protein
CXCL	C-X-C motif chemokine
CXCR	Chemokine receptor
DAG	1,2-diacylglycerol
DAPK	Death associated protein kinase
Daxx	Death-associated protein 6
DD	Death domain
DISC	Death-inducing signalling complex
EGFR	Epidermal growth factor receptor
eNOS	Nitric oxide synthase
EPAC	PKA independent exchange protein directly activated by cAMP
ERK	Extracellular regulated kinase
FADD	Fas-associated death domain protein
Fas/CD95	TNF receptor superfamily, member 6/cluster of differentiation 95
GAP	GTPase activating protein
GCK	Geminal center kinase

GDI	GDP-dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GLK	GCK-like kinase
GPCR	G-protein coupled receptor
Grb-2	Growth factor receptor-bound protein 2
GSH	Glutathione sepharose beads
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HCN	Cyclic nucleotide-gated channel
HEK293	Human embryonic kidney 293
Her	Human epidermal growth factor
HIF-1α	Hypoxia inducible factor 1-α
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon-gamma
IGF-BP	Insulin-like growth factor-binding protein
IκB-α	Inhibitory κB -α
IKK	IκB kinase
IL	Interleukin
IL-1R	IL-1 receptor
IL-1RAcP	IL-1 receptor accessory protein
IL-Ra	IL-1 receptor antagonist
IP_3	Inositol 1,4,5-trisphosphate
IRAK	IL-1 receptor associated kinase
JNK	c-Jun NH_2-terminal kinase
MADD	MAPK kinase activating-death domain
MAPK	Mitogen-activated protein kinase
MAPKK/MEK	MAPK kinase
MAPKKK/MEKK	MAPK kinase kinase
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemoattractant protein-1
MMP	Metalloproteinase
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHERF1	Na^+/H^+ exchanger regulatory factor
NPP	Nucleotide pyrophosphatase/phosphodiesterase
NTPDase	Ecto-nucleoside triphosphate diphosphohydrolase
oLDL	Oxidised low-density lipoprotein
P2YR	Purinergic receptors
PAR	Proteinase activated receptor

PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PEA-15	Phosphoproteins enriched in astrocytes 15
PECAM-1	Platelet endothelial cell adhesion molecule-1
PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinases
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PLCβ	Phospholipase C
PLD	Phospholipase D
PLAD	Pre-ligand-binding assembly domain
PMA	Phorbol 12-myristate 13-acetate
PUMA	p53 up-regulated modulator of apoptosis
RAFTK	Related adhesion focal tyrosine kinase
RAGE	Advanced glycation end products
RhoA	Ras-homology protein
RIP	Receptor interacting protein
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SAPK	Stress activating protein kinases
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
SOS	Son of sevenless
TAB	TAK binding protein
TAK	Transforming growth factor β-activated kinase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TNFR	TNF receptor
Tollip	Toll-interacting protein
TRADD	TNFR1 associated death domain
TRAF	TNF-receptor-associated factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Trx	Thioredoxin
Ubc13	Ubiquitin-conjugating enzyme (E2)
UDP	Uridine diphosphate
Uev1A	Ubiquitin-like protein
UTP	Uridine-5'-triphosphate
UVC	Ultraviolet type C
VCAM-1	Vascular cell adhesion molecule-1

VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

List of amino acid

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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Chapter 1: General Introduction

1.0 General Introduction

Statistics from the World Health Organisation have revealed that cardiovascular diseases and cancer are the main causes of non-communicable disease deaths in people under the age of 70, contributing 39 % and 27 % mortality rate respectively in 2008 (Alwan, 2011). In order to cure these diseases, pharmaceutical companies are in a continuous search for new drugs with better therapeutic effectiveness and minimal side effects. Numerous studies over the years have proven that the dysregulation of intracellular signalling pathways mediate the pathogenesis of several inflammatory-related diseases. One such pathway is the mitogen-activated protein kinase (MAPK). Hence, inhibition of one of these kinases, namely the pro-inflammatory c-Jun NH_2-terminal-kinase (JNK), could be an effective site of therapeutic intervention. In this study, the inhibition of inflammatory mediators driven JNK signalling via the activation of purinoceptors, a class of G-protein coupled receptor (GPCR), was examined in endothelial and cancer cells. The subsequent section first provides a brief overview of the characteristics of MAPK signalling, followed by the pathogenesis of atherosclerosis and cancer and the role of MAPK in these diseases.

1.1 MAPK signalling: An overview

In general, signalling through the classical MAPK cascade is composed of three sequentially activated serine-threonine specific protein kinases, which confer a vital role in cell function, proliferation and survival. There are four main pathways in the MAPK cascade that undergo a common phosphorylation sequence of MAPK kinase kinase (MAPKKK/MEKK) → MAPK kinase (MAPKK/MEK) → MAPK; namely extracellular regulated kinase 1/2 (ERK 1/2), ERK5, p38 MAPK and c-Jun NH_2-terminal kinase (JNK). These MAPKs undergo dual phosphorylation on threonine and tyrosine residues for their activation (Derijard et al., 1995). The activity of MAPK is modulated via interactions with scaffold proteins, docking site motifs, phosphatases and negative feedback interactions by other signalling pathways (Rose

et al., 2010). A summary of the signalling components involved in the four main pathways is shown in Figure 1.1.

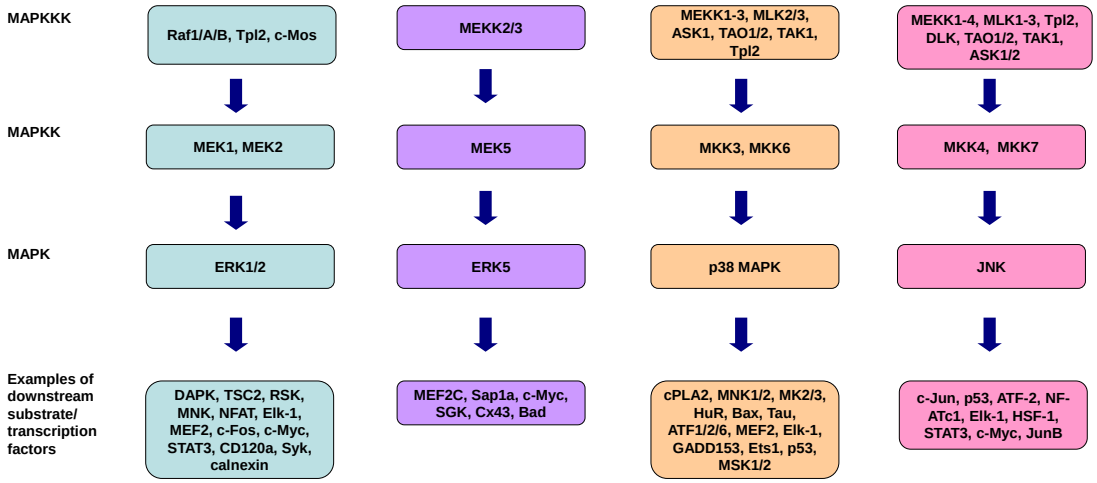


Figure 1.1: Summary of MAPK signalling cascade

Upstream signalling components and downstream activators involved in the MAPK signalling cascade of ERK1/2, ERK5, p38 MAPK and JNK are summarised above (Cargnello and Roux, 2011). MAPK: Mitogen-activated protein kinase; MAPKK/MEK: MAPK kinase; MAPKKK/MEKK: MAPK kinase kinase; ERK: Extracellular regulated kinase; JNK: c-Jun NH_2-terminal kinase; Tpl2: Tumour progression locus 2; DAPK: Death-associated protein kinase; TSC2: Tuberous sclerosis protein 2; RSK: p90 ribosomal protein S6; MNK: MAPK-interacting protein; NFAT: Nuclear factor of activated T cells; Elk-1: E twenty-six like transcription factor 1; STAT3: Signal transducer and activator of transcription 3; Syk: Spleen tyrosine kinase; MEF2C: Myocyte-specific enhancer factor 2C; Sap1a: Serum response factor accessory protein-1a; SGK: Serum- and glucocorticoid-inducible kinase; Cx43: Connexin 43; BAD: Bcl-2-associated death promoter; MLK: Mixed-lineage kinase; ASK1: Apoptosis signal regulating kinase-1; TAO: Thousand-and-one amino acid; TAK1: Transforming growth factor β-activated kinase 1; cPLA2: Cytosolic phospholipase A2; MK: MAPK-activated protein kinase; Bax: Bcl-2 associated X protein; ATF: Activating transcription factor; GADD: Growth arrest and DNA damage; Ets: E26 transformation specific sequence; MSK1/2: Mitogen- and stress-activated protein kinase 1/2; DLK: Dual leucine zipper-bearing kinase; NF-ATc1: Nuclear factor of activated T-cells, cytoplasmic 1; HSF-1: Heat shock factor protein 1.

1.1.1 Extracellular regulated kinase 1/2 (ERK 1/2)

The first discovered MAPK, ERK1/2 (Boulton et al., 1991a, Boulton et al., 1991b) plays a vital role in cell proliferation, differentiation and development. ERK1 and ERK2 are ubiquitously expressed and share 83% sequence homology. These isoforms may have a slightly different physiological role, as reflected in the fact that $ERK1^{-/-}$ mice are phenotypically normal, but ERK2 deletion results in embryonic lethality (Hatano et al., 2003, Selcher et al., 2001). Various growth factors (nerve growth factor, platelet derived growth factor and epidermal growth factor), insulin, GPCRs and cytokines can phosphorylate ERK1/2 (Cargnello and Roux, 2011). The majority of these extracellular stimuli induce the small G protein Ras to recruit Raf to the plasma membrane, allowing subsequent MEK1/2 and ERK1/2 phosphorylation (Tamada et al., 1997). Approximately 200 downstream effectors are linked to activation of ERK1/2, including phospholipase A_2, E twenty-six-like transcription factor 1 (*Elk1*), *c-Fos* and *c-Jun* (Wortzel and Seger, 2011).

Importantly, the physiological outcome of ERK1/2 signalling (referred as ERK for the rest of the thesis) is determined by the duration, magnitude and nuclear/cytoplasmic localisation of phosphorylated ERK (Ebisuya et al., 2005). For instance, although both transient and sustained ERK signalling could induce the transcription of early immediate genes, such as *Fos*, *Jun*, *Myc* and *Egr1*, only sustained ERK activity allows the stabilisation of encoded mRNA and protein level (Ebisuya et al., 2005). Sustained ERK activation increases cyclin D1 promoter activity and maintains its protein expression throughout the G1 phase to enable the S phase transition in the cell cycle for cell proliferation (Lavoie et al., 1996, Yamamoto et al., 2006). Conversely, phosphorylated ERK can be retained in the cytoplasm via binding to Sef, phosphoproteins enriched in astrocytes 15 (PEA-15) and through phosphorylation of death associated protein kinase (DAPK) which prevents the transcription of pro-survival genes in the nucleus (Chen et al., 2005, Formstecher et al., 2001, Torii et al., 2004).

1.1.2 p38 MAPK

Examination of kinase cascades activated by hyperosmolarity and endotoxins including lipopolysaccharide and the cytokine interleukin-1 led to the identification of another MAPK cascade, p38α MAPK (Han et al., 1994). Later, additional p38 MAPK isoforms were cloned, namely p38β (Jiang et al., 1996), p38γ (Li et al., 1996) and p38δ (Jiang et al., 1997). However their tissue expression pattern is different: p38α and β isoforms are expressed ubiquitously; p38γ is only found in skeletal muscle; and p38δ is prevalent in lung and kidney cells (Jiang et al., 1997). p38 MAPK is activated by both MKK3 (Derijard et al., 1995) and MKK6 (Han et al., 1996) to induce nuclear translocation of p38 MAPK for activation of transcription factors, such as ATF2, MNK1, p53 and Elk-1 (Raingeaud et al., 1995, Roux and Blenis, 2004).

1.1.3 c-Jun NH_2-terminal kinase (JNK)

An additional MAPK family, designated as JNK, has also been identified as a pathway activated in response to stress, cytokines, growth factors and GPCRs. These include ultraviolet (UV) irradiation (Hibi et al., 1993), hyperosmolarity (Bogoyevitch et al., 1995), anisomycin (Bogoyevitch et al., 1995), heat shock (Adler et al., 1995b), tumour necrosis factor-α (TNF-α) (Westwick et al., 1994), interleukin-1 (Gupta et al., 1996), interleukin-3 (Kendrick et al., 2004), colony-stimulating factor (Kendrick et al., 2004), epidermal growth factor (Westwick et al., 1994) and platelet derived growth factor (Assefa et al., 1999). Three JNK genes have been cloned, given rise to three isoforms; JNK1 (Derijard et al., 1994) and JNK2 (Kallunki et al., 1994) which are ubiquitously expressed, while JNK3 is mainly expressed in the brain, heart and testis (Gupta et al., 1996). From these isoforms, ten splice variances of JNK are expressed as 46, 48, 55 or 57 kDa proteins in different tissues (Gupta et al., 1996).

Depending on the stimuli, JNK could be activated by MKK4 (Derijard et al., 1995) and/or MKK7 (Moriguchi et al., 1997). TNF-α utilises MKK7 only for JNK activation but other JNK inducers, such as sorbitol and anisomycin, activate MKK4

and MKK7 for JNK activation (Moriguchi et al., 1997). Activation of JNK then leads to downstream phosphorylation of c-Jun that increases c-Jun-DNA binding and stabalises c-Jun for AP-1 activation (Musti et al., 1997, Papavassiliou et al., 1995). In contrast to AP-1 transcription factor activation, prolonged JNK signalling leads to apoptosis. In various cell lines, including Jurkat and fibroblast cells, sustained JNK signalling produces cell death as measured by DNA fragmentation (Chen et al., 1996b, Ventura et al., 2006). Other mechanisms regarding cytokine-induced apoptosis are discussed in section 1.4.

Growing evidence using specific JNK isoform knockout mouse models or siRNA suggest a differential role for JNK1 and JNK2 in the pathogenesis of diseases, including obesity, insulin resistance, diabetes, T cell differentiation, atherosclerosis and tumour development (Jaeschke et al., 2005, Yang et al., 1998, Sabapathy et al., 2004, Tuncman et al., 2006). For instance, $JNK1^{-/-}$ mice, but not $JNK2^{-/-}$, exhibit reduced adipose tissue and increased insulin sensitivity (Hirosumi et al., 2002). However cross-talk between these two JNK isoforms has been suggested as both $JNK1^{-/-}JNK2^{+/+}$ and $JNK1^{+/+}JNK2^{-/-}$ mice demonstrated similar reduction in serum insulin and glucose levels compared to wild type (Tuncman et al., 2006). Nevertheless, these studies suggest that selective inhibition of JNK isoforms in disease could be desirable.

1.2 The role of MAPK in disease: Atherosclerosis

1.2.1 An overview for the role of endothelial cells in atherosclerosis

Atherosclerosis is characterised by the formation of fatty lesions at the blood vessel inner lumen which ultimately detach from the wall to obscure the blood supply to the heart, leading to heart ischemia and infarct (Ross, 1999). Various cell components such as vascular smooth muscle cells (VSMC), endothelial cells and circulating immune cells work cooperatively in the progression of the disease (Ross, 1999, Hansson and Hermansson, 2011). In particular, endothelial dysfunction is implicated as an initiator of the disease (Endemann and Schiffrin, 2004, Esper et al., 2006). Early understanding of endothelial dysfunction is characterised by a defect in vasodilation in blood vessels in response to acetylcholine (Ludmer et al., 1986), whilst later studies have shown its wider definition of 'the partial or complete loss of balance between vasoconstrictors and vasodilators, growth promoting and inhibiting factors, pro- and anti-antherogenesis factors' (Quyyumi, 1998). Various risk factors, including hyperlipidemia, hypertension, diabetes, obesity, aging and smoking, could lead to an increase in reactive oxidative species followed by a decrease in nitric oxide production and an increase in pro-inflammatory mediator expression (Higashi et al., 2009).

Vascular injury initiated by inflammatory cytokines or chemokines, reactive oxygen species and oxidised lipoproteins stimulate the activation of signalling pathways in endothelial and smooth muscle cells to increase the expression of inflammatory mediators (Libby et al., 2002, Patel et al., 2000, Hansson and Hermansson, 2011). These inflammatory mediators play an important role in the early phase of atherosclerosis lesion formation via the tethering, rolling, adherence and transmigration of monocytes and leukocytes into the subendothelial space (Blankenberg et al., 2003, Libby, 2006). Vascular cell adhesion molecule-1 (VCAM-1) along with P- and E- selectin are involved in the first step of tethering and rolling, followed by intercellular adhesion molecule-1 (ICAM-1) to induce firm adhesion and platelet endothelial cell adhesion molecule-1 (PECAM-1) to stimulate immune cell migration. Besides these adhesion molecules, other mediators also aid the formation

of foam cells, including monocyte chemoattractant protein-1 (MCP-1) which enhances recruitment of more monocytes into the lesion and macrophage colony-stimulating factor (M-CSF) which promotes the transformation and proliferation of macrophages. The maturation of monocytes into macrophages form a positive feedback loop in the recruitment of immune cells through the release of pro-inflammatory cytokines, such as TNF-α and interleukin-1β (IL-1β). Metalloproteinases (MMPs) then degrade the collagen in the extracellular matrix of the atherosclerotic lesion, rupturing the plaque to form a thrombus (Ross, 1999). Details of the inflammatory mediators that promote atherosclerosis progression are summarised in Figure 1.2 below.

Although inflammation is the main feature of atherosclerosis, cell apoptosis is also prominent in atherosclerotic lesions. One study has shown an increase in TUNEL staining in the atherosclerotic plaque, mainly in macrophages and T cells (Bjorkerud and Bjorkerud, 1996). Apoptosis, mediated by the activation of the caspase cascade, determines the stability of the atherosclerotic plaque (Kockx and Herman, 2000, Rossig et al., 2001). During apoptosis, endothelial cells exhibit pro-coagulation characteristics with increased factor Xa and tissue factor activity that increase platelet binding, causing thrombus formation (Bombeli et al., 1997, Bombeli et al., 1999, Greeno et al., 1996). Collectively, endothelial cells have a major role in the initiation and modulation of atherosclerosis through inflammation and apoptosis.

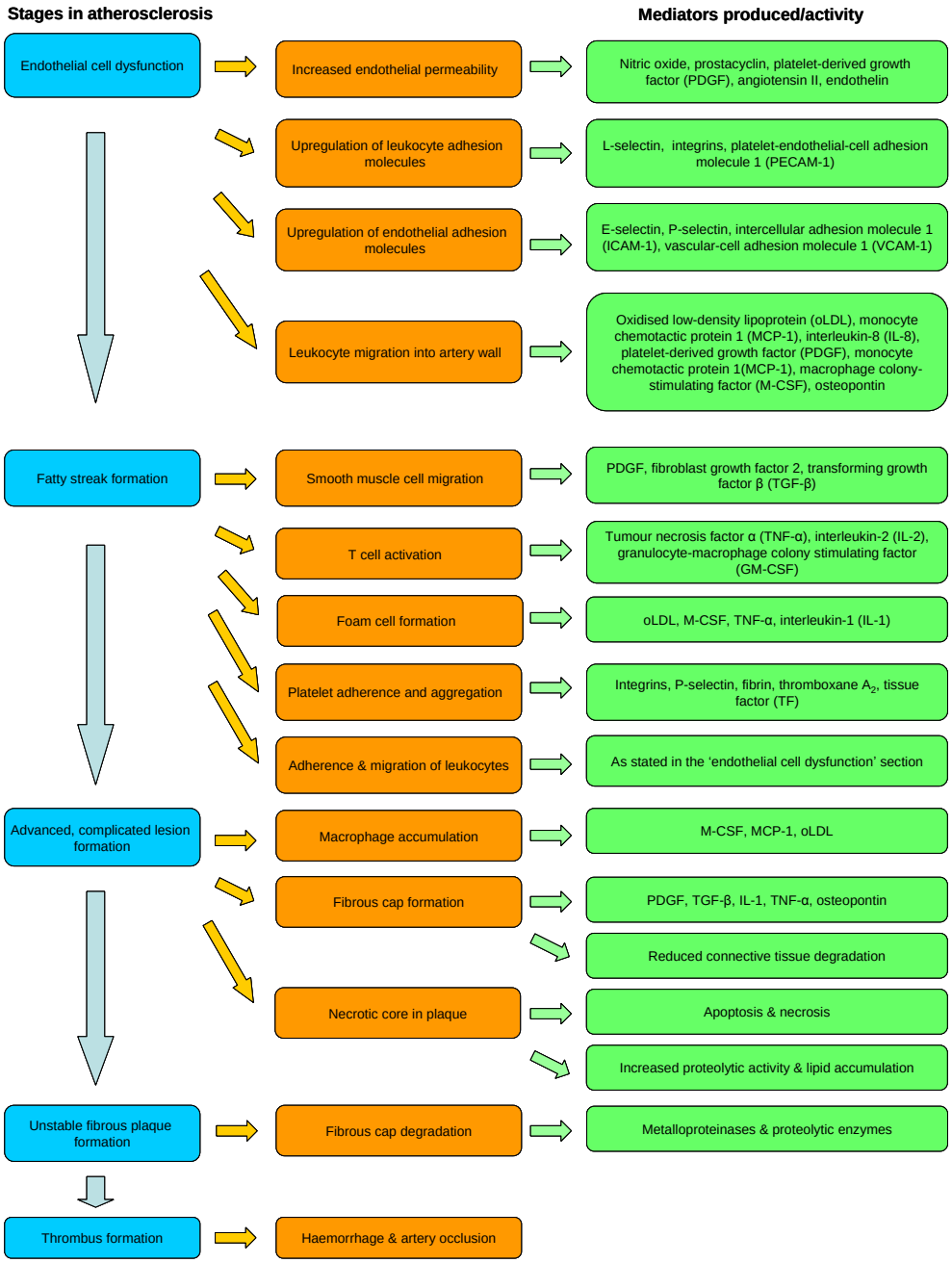


Figure 1.2: Various stages in atherosclerosis and the mediators involved

Progression of the atherosclerotic plaque involves initial endothelial dysfunction due to a combination of factors including elevated plasma levels of LDL, homocysteine and free radicals. These factors trigger the secretion of inflammatory mediators which in turn drive the formation of fatty streak, lesions and thrombus (Ross, 1999, Esper et al., 2006).

1.2.2 The role of MAPK in atherosclerosis

ERK signalling: Numerous studies have shown that each MAPK plays a different role in the pathogenesis of atherosclerosis. An increase in ERK phosphorylation has been observed in atherosclerotic lesions in arteries derived from human patients, cholesterol-fed rabbits and pigs post-balloon angioplasty (Anger et al., 2008, Hu et al., 2000, Liu et al., 2002) and is associated with an increase in neointima formation in atherosclerosis and after vascular injury (Gennaro et al., 2003). Application of an ERK inhibitor delivered orally to rats with balloon injury successfully reduced cell proliferation and vessel intima/media ratio via cell cycle G1 arrest but not increase in apoptosis (Gennaro et al., 2004). Cellular studies support the results obtained *in vivo*, pro-atherosclerotic oxidised low-density lipoprotein (oLDL) and platelet-derived growth factor (PDGF) have been found to be potent activators of ERK to induce VSMC proliferation (Yang et al., 2000, Yang et al., 2001a, Gennaro et al., 2003, Zhan et al., 2003). Detailed studies have also shown that oLDL stimulates ERK activity in a PKC and Ca^{2+} dependent mechanism (Yang et al., 2000, Yang et al., 2001a). ERK signalling also mediates the PDGF driven gene expression in VSMC as dominant-negative ERK reduced PDGF mediated plasminogen activator inhibitor type-1, MCP-1 and transforming growth factor-β1 expression (Zhan et al., 2003). These studies collectively suggested that ERK inhibitors could be of therapeutic use in the treatment of atherosclerosis or post cardiac procedures.

p38 MAPK signalling: In general, p38 MAPK is strongly linked to inflammation in a number of tissues and is implicated in the development of atherosclerosis. Various *in vitro* studies have demonstrated that p38 MAPK mediates the expression of inflammatory mediators that are known to promote neutrophil migration into the atherosclerotic lesion (Pietersma et al., 1997, Wang and Doerschuk, 2001). A decrease in TNF-α induced VCAM-1 expression at the endothelial cell surface was observed following p38 MAPK inhibition, in the absence of any effect in mRNA levels (Pietersma et al., 1997), suggesting that p38 MAPK modulates VCAM-1 expression via a post-translation mechanism. Furthermore p38 MAPK inhibition prevented cross-linking of ICAM-1 by inhibiting the phosphorylation of heat shock

protein 27, an actin-binding protein in endothelial cells (Wang and Doerschuk, 2001). A recent study then reported another role for p38 MAPK in foam cell formation, as p38 MAPK inhibitor, SB203580, and siRNA both reduced autophagy-dependent LDL cholesterol ester accumulation in the macrophages, possibly through inhibiting the autophagy gene, unc-51-kinase 1 (Ulk1) (Mei et al., 2012).

These results obtained *in vitro* correlated with the findings observed *in vivo* using p38α$^{-/-}$ and apolipoprotein E$^{-/-}$ (ApoE$^{-/-}$) mouse models. Following oLDL stimulation, less VCAM-1, MCP-1, IP-10 and C-X-C motif chemokine 1 (CXCL1) were produced in endothelial cells derived from double p38α$^{-/-}$ ApoE$^{-/-}$ compared to ApoE$^{-/-}$ alone (Kardakaris et al., 2011). The pro-atherosclerotic role of p38 MAPK is also demonstrated in another *in vivo* model whereby ApoE$^{-/-}$ mice with selective macrophage deletion of p38α exhibited lesion instability with increased apoptosis, fibrous cap thinning and collagen depletion. This study demonstrated that p38α inhibition induced endoplasmic reticulum stress in the macrophage via suppression of Akt phosphorylation (Seimon et al., 2009). Even though the necrosis, collagen and foam cell content in the atherosclerotic lesions in p38α$^{-/-}$ ApoE$^{-/-}$ was not significantly different compared to ApoE$^{-/-}$ alone (Kardakaris et al., 2011), pharmacological inhibition of p38 MAPK could still be therapeutically beneficial as treatment of ApoE$^{-/-}$ mice with the p38 MAPK inhibitor, SB203580, for 4 months reduced the atherosclerotic lesion size by approximately half (Seeger et al., 2010).

JNK signalling: Various studies have shown the activation of JNK signalling in the vascular wall (endothelial cells and VSMC) during injury, including atherosclerosis, abdominal aortic aneurysm (AAA) and arterial restenosis post balloon angioplasty (Izumi et al., 2001, Hahn et al., 2009, Metzler et al., 2000, Yoshimura et al., 2005). In the atherosclerosis samples from animal models of ApoE$^{-/-}$ and cholesterol fed rabbits, elevated JNK phosphorylation at the atherosusceptible site is colocalised with the expression of pro-inflammatory proteins (ICAM-1 and VCAM-1) and pro-apoptotic molecules (p53, pro-apoptotic Bax and Bcl-X) respectively (Metzler et al.,

2000, Orr et al., 2005, Wang et al., 2011). Inhibition of JNK in $ApoE^{-/-}$ mice also reduces substantially the presence of atherosclerotic lesions (Wang et al., 2011). Interestingly, several studies have suggested a role for JNK as a positive regulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling as a JNK inhibitor reduced phosphorylation and expression of p65 NF-κB (Wang et al., 2011, Cuhlmann et al., 2011). In mouse models of AAA and restenosis, abrogation of JNK activity via pharmacological inhibition and dominant-negative JNK expression successfully prevented the development of these respective phenotypes (Izumi et al., 2001, Yoshimura et al., 2005).

Further studies have also illustrated the important role of JNK signalling in mediating the pro-atherosclerotic effect of TNF-α. Results from microarray, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and Western blotting on human umbilical vein endothelial cells (HUVECs) have shown that TNF-α stimulated higher expression of VCAM-1, E-selectin, interleukin-8, caspase 3 and protein phosphatase A (PP2A), toll-like receptor 4 (TLR4) and receptor interacting protein-1 (RIP1) which is reversed by the JNK inhibitor CT536706 (Chaudhury et al., 2010). TNF-α also promotes lesion destabilisation by decreasing the mRNA expression of pro-collagen enzyme subunit prolyl-4-hydroxylase αI (P4Hα(I)) which is abolished in the presence of a JNK inhibitor (Zhang et al., 2007). In addition, the reduction of TNF-α mediated nitric oxide (NO) release and vasodilatation in response to adenosine observed in pig coronary arterioles is reversed through pre-incubation with a JNK inhibitor (Zhang et al., 2006).

Another enzyme of the arachidonic acid pathway, namely cyclooxygenase-2 (COX-2), that produces downstream prostanoids such as prostaglandin E2 (PGE_2) and prostacyclin, is also found to be upregulated in atherosclerotic lesions in a JNK dependent manner (Belton et al., 2000, Cipollone et al., 2005a). Recent studies have demonstrated a pro-atherogenic role of COX-2 through the activation of EP4 receptor by PGE_2, in order to increase the expression of IL-6, metalloproteinases

(MMP) 2 and 9 (Cipollone et al., 2005b, Yokoyama et al., 2012). However application of a COX-2 inhibitor (MF-tricyclic and nimesulide) to ApoE$^{-/-}$ and LDL receptor $^{-/-}$ mice for 3 weeks and 18 weeks produced increased and decreased atherosclerotic lesion size respectively (Pratico et al., 2001, Rott et al., 2003). These findings suggested that prolonged COX-2 inhibition mediated by JNK inhibition could be therapeutically beneficial.

Other studies have also demonstrated that JNK activity could positively modulate the expression levels of cytokine signalling components, such as receptor-interacting protein 1 (RIP1) and pro-caspase 3, hence creating a positive loop of inflammation (Chaudhury et al., 2010). Oxidised LDL could activate TLR4 for increased NF-κB signalling (Yang et al., 2005) leading to increase in IL-1β promoter activity (Hiscott et al., 1993), inflammasome NOD-like receptor family and pyrin domain containing 1 (NLRP-1) expression in endothelial cells (Yin et al., 2009). Elevated secretion and processing of IL-1β leads to more inflammatory activity via greater IL-1 receptor I (IL-1RI) stimulation.

Furthermore, there are a few studies utilising knockout mice models which have suggested an isoform selective role for JNK in atherosclerosis. Compared to mice deficient in ApoE alone (ApoE$^{-/-}$), JNK2$^{-/-}$ ApoE$^{-/-}$, but not JNK1$^{-/-}$ ApoE$^{-/-}$ develop lesser lesions (Ricci et al., 2004). This study also demonstrated that JNK2$^{-/-}$ macrophage have reduced foam cell formation via a decrease in modified lipoprotein (acetylated and oxidised LDL) uptake, with increase in expression and reduced phosphorylation of macrophage scavenger receptor A (SR-A) (Ricci et al., 2004). JNK2$^{-/-}$ mice fed on a high cholesterol diet are also protected from endothelial dysfunction as hypercholesterolemia mediated reduction in NO, anti-oxidant superoxide dismutase (EC-SOD) and manganese superoxide dismutase (Mn-SOD) activity, compared to wild type (Osto et al., 2008). Thus, selective inhibition of JNK2 could be useful in atherosclerotic therapy.

1.3 The role of MAPK in disease: Cancer

1.3.1 An overview of inflammation in cancer development

Cancer, described as an aberrant, uncontrolled growth of cells, arises as a result of genetic alteration. Cancer leads to the development of a tumour, consisting of multiple cell types, such as cancer cells, immune cells, fibroblasts and endothelial cells, that collectively create a microenvironment conducive for tumour growth (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011, Pietras and Ostman, 2010). The characteristics of cancer are summarised as evading apoptosis, self-sufficiency in growth signals, tissue invasion and metastasis, insensitivity to anti-growth signals, sustained angiogenesis and limitless replicative potential (Hanahan and Weinberg, 2000). Latterly, inflammation has been added on as one of the hallmarks of cancer (Colotta et al., 2009, Hanahan and Weinberg, 2000), indicating that pro-inflammatory cytokines, chemokines and leukocyte infiltration also play an important role in cancer cell survival and invasion (Dinarello, 2006).

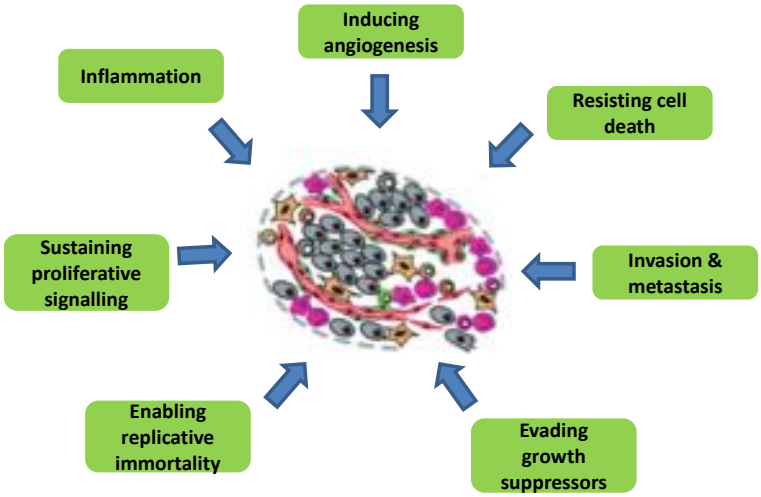
Despite the majority of studies focusing on the cellular proliferation in cancer, early studies examining various cancers, including lung, anal, oesophagus and breast, noted that chronic inflammation is commonly observed (Esipova, 1951, Gabriel, 1941, Kilgore and Fleming, 1952, Moreno, 1950). The inflammation phenotype of cancer cells induces the recruitment of diverse leukocytes into the tumour environment, which then create a positive feedback loop of chemokine and cytokine secretion within the tumour microenvironment (Coussens and Werb, 2002). For instance, tumour-associated macrophages secrete an array of pro-cancerous factors, such as vascular endothelial growth factor (VEGF), MMP, CXCL8, TNF-α, IL-6 and IL-10, to support cancer cell growth, tumour migration and angiogenesis (Allavena et al., 2008, Schoppmann et al., 2002, Mantovani et al., 2009). Evidence also suggests that cancer cells could modulate the activity of immune cells as macrophages secrete more TNF-α upon exposure to the supernatant of breast cancer cells, which in turn increases E-selectin expression to support metastasis (Eichbaum et al., 2011). Cytokines can also modulate the activity of other growth factors as IL-1β induces G1 cell arrest in breast cancer cells through inhibition of insulin-like growth factor-I

(IGF-I) signalling, including insulin receptor substrate-1 phosphorylation, cyclin-dependent kinase 2 (Cdk2) activation and enhanced cyclin A expression (Shen et al., 2004).

An *in vivo* model has also shown that certain cytokines and chemokines are pro-cancerous. Injection of B16 melanoma cells into IL-1$\beta^{-/-}$ mice have impaired angiogenesis, leading to less cancer development and improved survival rates (Voronov et al., 2003). Furthermore, implantation of Lewis lung carcinoma cells transfected with IL-1β has resulted in greater tumour volume and vascularisation due to elevated VEGF and MIP-2 (mouse homolog for IL-8) (Saijo et al., 2002). The pro-cancerous role of CXCR7 is suggested in a similar xenograft study whereby implantation of MDA-MB-435 overexpressing CXCR7 demonstrated greater tumour volume compared to wild type (Miao et al., 2007).

These laboratory studies correlate well with the clinical data as results derived from microarray and SAGE (serial analysis of gene expression) revealed that cancer tissues from patients exhibit different cytokine expression profiles compared to normal tissues (Allinen et al., 2004, Carlsson et al., 2011). Immunohistochemical analysis of patient breast cancer cells also showed an elevation in MCP-1, CXCL5, CXCL12, CXCL14, TNF-α and IL-1β (Soria et al., 2011, Allinen et al., 2004). An increase in cytokine levels was not confined to the tumour microenvironment, but also occurred in the plasma and indeed may be useful biomarkers for cancer. For example, elevated plasma acute phase protein (APP) and interleukin-6 (IL-6) were found to be associated with disease recurrence and poorer prognosis in breast cancer (Cole, 2009, Salgado et al., 2003, Zhang and Adachi, 1999). Thus, inhibition of cytokine and chemokines could be a promising therapeutic target in cancer as evaluated in clinical trials (Lazennec and Richmond, 2010).

A



B

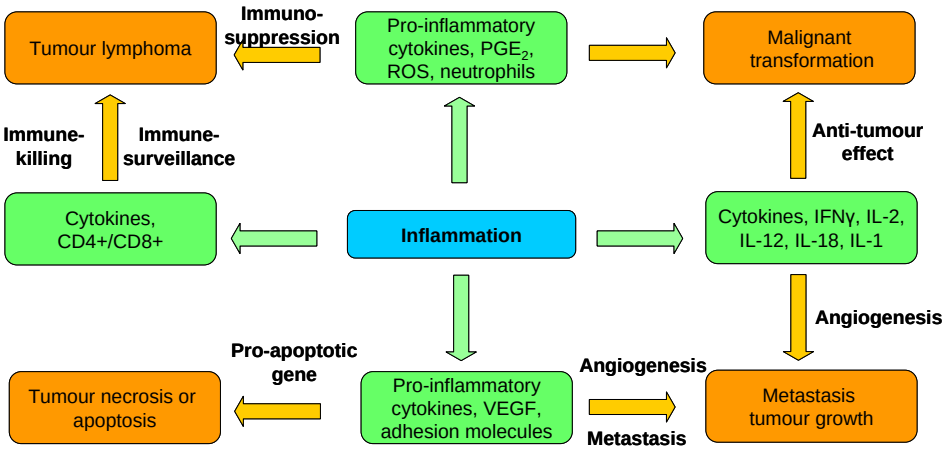


Figure 1.3: Hallmarks of cancer and the relationship between inflammation and cancer

Tumours consist of various cell types including cancer cells, immune cells, fibroblasts and endothelial cells that form a tumour microenvironment to support its growth. The seven characteristics of cancer are also highlighted in green in (A) whereas the inflammatory proteins that interact with cancer environment are stated in (B) (Hanahan and Weinberg, 2011, Dinarello, 2006). PGE_2: Prostaglandin E_2; ROS: Reactive oxygen species; IFNγ: Interferon-gamma; IL: Interleukin, VEGF: Vascular endothelial growth factor.

1.3.2 The role of MAPK in cancer

MAPK signalling in inflammation is associated with cancer: As discussed above, a reduction in inflammation within cancer could be achieved by decreasing the expression or activity of cytokines and chemokines. However, most studies in cancer have focused on cytokine and chemokine receptor inhibitors, thus inhibition of MAPK in inflammation linked to cancer is largely extrapolated from other inflammatory diseases. For instance, pharmacological inhibition of p38 MAPK in bone marrow stromal cells reduced TNF-α induced IL-6 secretion (Hideshima et al., 2003), whereby IL-6 is generally found to promote cancer cell growth and metastasis (Hideshima et al., 2003, Schafer and Brugge, 2007, Tawara et al., 2011).

The limited studies in cancer cells have also supported the pro-cancerous role of MAPK signalling in relation to the secretion of inflammatory mediators. Activation of ERK is well known to increase the expression of IL-8 in various cancer cells to promote vascularisation and endothelial cell recruitment (Sparmann and Bar-Sagi, 2004). In colon cancer cells, inhibition of ERK, p38 MAPK and JNK signalling using pharmacological inhibitors have been shown to reduce IL-1β induced COX-2 expression, which could decrease the proliferative capacity of COX-2 in cancer cells (Oshima et al., 1996, Liu et al., 2003). Inhibition of JNK using siRNA also reduced pancreatic cancer cell migration stimulated by IL-1β as shown by a wound healing assay (Verma et al., 2012). In prostate cancer cells, IL-4 induced a proliferative effect in a JNK-dependent manner, whereas p38 MAPK negatively regulated TNF-α induced apoptosis (Roca et al., 2012, Ricote et al., 2006). Inhibition of p38 MAPK also decreased the secretion of G-CSF, IL-6, IL-10, MCP-5, CCL5, TNF-α and VEGF in dendritic cells treated with tumour culture conditioning medium (Wang et al., 2006).

MAPK signalling could also be implicated in the side-effects of chemotherapeutic agents. Inhibition of p38 MAPK can reduce the cytokine-induced fatigue during chemotherapy as the increase in IL-1β, TNF-α and/or IL-6 expression, but not the

cytotoxic effects of etoposide, 5-fluorouracil and doxorubicin is reversed in the presence of a p38 MAPK inhibitor, ML3404 (Elsea et al., 2008). In addition to inflammation, MAPK signalling also contributes to the pathogenesis of cancer via direct modulation of cell growth, apoptosis and migration (as discussed below), however the studies highlighted above do suggest that the inflammatory driven responses mediated by MAPK can play a role in cancer.

ERK signalling: Since the initial discovery of the Ras proto-oncogene being a key regulator of ERK signalling, a large body of evidence has accumulated, implicating the MAPK pathway in the pathogenesis of cancer. Hyperactivation of the ERK pathway, either by mutation of its upstream intermediates Raf or Ras, causes uncontrolled cell proliferation and chemotherapeutic resistance in various cancers, such as melanoma, breast and thyroid cancer (McCubrey et al., 2007, Sheridan et al., 2008, Karasarides et al., 2004). Mutation of Ras is found in up to 30% of all cancers, but is more prevalent in pancreatic, colon, thyroid, lung and melanoma cancers (Bos et al., 1987, Davies et al., 2002). Activation of ERK promotes cancer cell survival by phosphorylation of B-cell lymphoma 2 (Bcl2) protein family, including Bcl-2-like protein 1-extra long (Bim_{EL}), Bcl-2-associated death promoter (Bad) and myeloid cell leukemia sequence 1 (MCL-1), to reduce pro-apoptotic signals (Domina et al., 2004, Fueller et al., 2008, Ley et al., 2003). Activation of ERK is also implicated in cancer cell motility and invasion through stabilisation of Fos-related antigen-1 (Fra-1) transcription factor expression to suppress β1-integrin signalling to Rho in colon cancer cells (Vial et al., 2003, Vial and Marshall, 2003). However some evidence also suggests a pro-apoptotic role for ERK signalling as DNA damaging stimuli such as etoposide, UV and ionising irradiation can also activate ERK. Inhibition of ERK signalling reduces the apoptotic effect of these stimuli, but via a mechanism independent of p53 activity (Tang et al., 2002). In summary, ERK activation could lead to diverse effects on cell growth depending on the stimuli and cell type studied.

p38 MAPK signalling: In contrast to ERK, p38 MAPK activation is largely associated with a negative effect upon cell proliferation and survival (Bradham and

McClay, 2006, Xia et al., 1995, Olson and Hallahan, 2004). Deletion of both upstream kinases, MKK3 and MKK6, induces uncontrolled proliferation and tumour formation in mice (Brancho et al., 2003). These results are consistent with the ability of p38 MAPK to decrease cyclin D expression and the phosphorylation of p53 at Ser33 and Ser46, cdc25B at Ser 309 and Ser 361 and cdc25C at Ser 216 (Bulavin et al., 2001, Bulavin et al., 1999, Lavoie et al., 1996). On the other hand, p38 MAPK signalling can also produce a pro-apoptotic outcome as its activation by high concentrations of TNF-α and UVC mediates the internalisation of EGFR to induce apoptosis (Zwang and Yarden, 2006). Activated p38 MAPK also phosphorylates p53 at Ser33 and Ser46 to mediate the apoptotic effect of p53 (Bulavin et al., 1999). One study suggests that the pro-apoptotic effect of p38 MAPK is linked to cellular ERK expression, high ERK activity relative to p38 MAPK is found in proliferating cells whereas a low ERK to p38 MAPK activity ratio is detected in dormant cells (Aguirre-Ghiso et al., 2004). Pharmacological inhibition of p38 MAPK could be applied concurrently with chemotherapy for higher therapeutic efficacy as it sensitises quiescent cells to fluorouracil and cytarabine induced apoptosis as observed in a leukemic cell model (Vaidya et al., 2008).

JNK signalling: Similar to other MAPKs, JNK has been found to be highly phosphorylated in various cancer tissues, including breast, glioblastoma and melanoma (Cui et al., 2006, Davidson et al., 2006, Gee et al., 2000, Lopez-Bergami et al., 2007, Wang et al., 2010b, Yeh et al., 2006). Although JNK activation was linked to a lower patient survival rate, the exact pro- or anti-apoptotic role of JNK in cancer has remained controversial, and could be cell type or JNK isoform dependent (Kennedy and Davis, 2003, Weston and Davis, 2007). Microarray analysis has demonstrated different patterns of gene expression in embryonic cells derived from $JNK1^{-/-}$ and $JNK2^{-/-}$ mice (Chen et al., 2002) whilst in mouse skin studies increased tumour formation has been found in $JNK1^{-/-}$ mice (She et al., 2002), but is reduced in $JNK2^{-/-}$ mice (Chen et al., 2001). However increased tumour proliferation following JNK2 deletion has been shown in other cell types, such as hepatocytes, fibroblasts and erythroblasts (Sabapathy and Wagner, 2004). These differences may be

clinically relevant as in breast cancer cells, high JNK2 expression was observed to negatively correlate with patient survival (Mitra et al., 2011). High JNK1 expression was also found in breast cancer tissues with overexpressed human epidermal growth factor 2 (Her2) and JNK1 inhibition induced apoptosis in cells derived from these tissues (Han and Crowe, 2010).

The studies outlined above conducted *in vivo* have been supported by a number of experiments performed in different cancer cell models. Work has largely focused on the regulation of apoptosis mediated principally by JNK1 or has not sort to distinguish the roles of the different JNK isoforms. Indeed, JNK has been linked to apoptosis, as characterised by DNA fragmentation, chromatin condensation, caspase 3 activation and membrane blebbing, via either the intrinsic pathway through nuclear translocation or the extrinsic pathway through activation of mitochondrial dependent caspases (Dhanasekaran and Reddy, 2008, Lei et al., 2002). Activation of JNK within the nucleus elevated the expression of pro-apoptotic proteins, such as TNF-α, TNF receptor superfamily, member 6 (Fas) and Bcl-2 homologous antagonist/killer (Bak) (Dhanasekaran and Reddy, 2008). Further detailed studies in Jurkat T cells has shown that JNK activation led to the binding of the transcription factors, ATF2 and c-Jun, to the Fas promoter region at position 338 and 316, to increase apoptosis (Faris et al., 1998, Faris et al., 1998(a)). JNK has also been shown to phosphorylate the transcription factor p53 at Thr81 to induce proliferation arrest through $p21^{Cip1/Waf1}$ and 14-3-3 activity and also apoptosis through Bax, Fas, Insulin-like growth factor-binding protein 3 (IGF-BP3) and p53 up-regulated modulator of apoptosis (PUMA) (Buschmann et al., 2001, Fuchs et al., 1998). Another member of the p53 family, p73, has also been found to be phosphorylated by JNK to stimulate apoptosis (Jones et al., 2007).

In addition to transcription factor induced apoptosis, JNK signalling can also modulate apoptosis via the mitochondria. In fibroblasts derived from $JNK^{-/-}$ mice, the apoptotic effect of UVC, DNA-alkylating agent methyl methanesulfate and

anisomycin is prevented due to a lack of mitochondrial depolarisation and cytochrome c release (Lei et al., 2002, Tournier et al., 2000). The mitochondrial pathway is regulated by several Bcl2 family proteins, including Bcl-2, Bcl-X_L and Mcl-1 which are anti-apoptotic, and Bax, Bad, Bak, BH3 interacting-domain death agonist (Bid) and Bim which are pro-apoptotic (Lucken-Ardjomande and Martinou, 2005, Scorrano and Korsmeyer, 2003). Activated mitochondrial JNK can phosphorylate Bcl-2 at Ser70, Ser87 and Thr69; and Bcl-X_L at Thr47 and Thr115 to inhibit their anti-apoptotic effect (Brichese et al., 2004, Kharbanda et al., 2000, Lei et al., 2002, Yamamoto et al., 1999). In addition, activated JNK can also phosphorylate BimL and Bmf to allow their dissociation from their binding partners myosin V motor complex and dynein motor complex respectively (Lei and Davis, 2003). Bim then activates Bax for the initiation of mitochondrial apoptotic pathway (Harris and Johnson, 2001, Marani et al., 2002). Activated JNK also phosphorylates 14-3-3ζ at Ser184 and 14-3-3σ at Ser186 to allow the dissociation of Bax from 14-3-3 binding, allowing the freed Bax to translocate into the mitochondria for cytochrome c release and apoptosis (Tsuruta et al., 2004). It should be noted that JNK signalling mediates the cell survival effects of interleukin-3 via phosphorylation of Bad at Thr201 to inhibit the association of Bad with BCL-X_L (Yu et al., 2004), suggesting that not all effects of JNK within the mitochondria are pro-apoptotic.

More recent studies have focused on a role for JNK2 in regulating proliferation and apoptosis. It has been demonstrated that in cancer cells, upregulation of JNK2 suppressed the pro-apoptotic pathway mediated by JNK1, thus siRNA for JNK2 induced apoptosis in various cancer cells lines via accumulation of c-Jun and down-regulation of the oncoprotein Bcl-3 (Ahmed and Milner, 2009). The expression of JNK2 also caused enhanced cell proliferation and migration through an increase in EGFR activation via the formation of adaptor proteins epidermal growth factor substrate 8 (EPS8)-Abl interaction-1 (Abi-1)-Son of sevenless-1 (Sos-1) (Mitra et al., 2011). In addition, JNK2 was observed to co-localise with DNA ligase in the nucleus following UVC dependent single stranded DNA damage, to allow cell cycle

progression (Chen et al., 2010). Thus, selective inhibition of JNK2 could be favourable in cancer therapy.

1.4 Activators of JNK in atherosclerosis and cancer

Given the importance of JNK in atherosclerosis and cancer, it is useful to describe the mechanisms through which endogenous activators regulate this pathway. Key inflammatory cytokines, include TNF-α and IL-1β whilst in the context of cancer, UVC is a noted activator, relevant primarily in skin cancer.

1.4.1 Cytokines as JNK activators

Inflammatory cells, such as macrophages and mononuclear cells, are the main contributors of cytokine production in both atherosclerosis and cancer conditions (Kishikawa et al., 1993, Tipping and Hancock, 1993, Lewis and Hughes, 2007, Tipping et al., 1993). In addition, endothelial cells and cancer cells themselves could also produce TNF-α and IL-1β in response to pro-inflammatory stimuli. For instance, lipopolysaccharide, advanced glycation end products (AGE) and specific forms of oLDL stimulate the secretion of TNF-α and IL-1β by endothelial cells (Lemaire et al., 1998, Miossec et al., 1986, Ranta et al., 1999, Rashid et al., 2004). The following sections will discuss the general characteristics of TNF-α and IL-1β, and the upstream signalling intermediates involved in JNK activation.

1.4.1.1 TNF-α

The pro-inflammatory cytokine TNF-α potently mediates both acute (Song et al., 2001b) and chronic inflammation, such as rheumatoid arthritis (Feldmann and Maini, 2001), postmenopausal osteoporosis (Roggia et al., 2001) and diabetes (Sethi et al., 2000). TNF-α cloned in 1984 is found to exist in both soluble (Pennica et al., 1984) and membrane-integrated forms (Kriegler et al., 1988). Further studies have identified other TNF superfamily members, amounting to a total of 19 TNF ligands and 30 corresponding receptors (Ware, 2008). TNF-α activates the ubiquitously

expressed TNF receptor 1 (TNFR1) and TNFR2 (Brockhaus et al., 1990), by inducing receptor trimerisation at the pre-ligand-binding assembly domain (PLAD) (Chan et al., 2000). However only the TNFR1 contains an intracellular death domain (DD) (Tartaglia et al., 1993) which allows recruitment of the adapter protein, named TNFR1 associated death domain (TRADD) (Hsu et al., 1995) upon receptor activation. Without a DD, TNFR2 forms a heterodimer with TNF-receptor-associated factor 2 (TRAF2) for signal transduction (Rothe et al., 1994).

1.4.1.1.1 Activation of JNK pathway by TNF-α

Since TNF-α is overexpressed in inflammatory conditions, its activation of stress activated protein kinases (SAPK) has been studied extensively, in particular JNK signalling. The initial step of TNFR1 activation involves receptor trimerisation, followed by the recruitment of TRADD, TRAF2 and receptor interacting protein (RIP) to the receptor complex (Chan et al., 2000, Hsu et al., 1996). Immunoprecipitation studies show that geminal center kinase (GCK) and GCK-like kinase (GLK) acts as signal intermediates to link TRAF2 to downstream MEKK1 (Diener et al., 1997, Yuasa et al., 1998). MEKK1 then phosphorylates MKK7 to activate JNK and AP-1 (Foltz et al., 1998, Moriguchi et al., 1997). In addition, one study in adipocytes reveals that TNF-α and TRAF2 can activate $G_{q/11}$ through β-arrestin-1, leading to cdc42/PI3K, MEKK1 and JNK activation (Kawamata et al., 2007), however this interaction has not been demonstrated in other cell types and is unlikely to be a bonafide pathway.

Even though TNF-α normally activates JNK only transiently, inhibition of cellular FADD-like IL-1β-converting enzyme inhibitory protein (c-FLIP) and NF-κB allows a prolonged activation of JNK by TNF-α (Nakajima et al., 2008, Wicovsky et al., 2007). Under these circumstances, JNK activity is found to be caspase and reactive oxygen species (ROS) dependent. Activation of JNK still requires the activity of upstream MEKK1 but the exact caspase isoform(s) involved remain unclear. Fas-associated death domain protein (FADD) binds to TNFR1, followed by downstream

caspase 8 and caspase 3 activation (Schneider-Brachert et al., 2004), these caspases may produce a persistent JNK signal. Furthermore ROS oxidises and dissociates the thioredoxin (Trx)-apoptosis signal-regulating kinase 1 (ASK1) complex, together with TRAF2, enables the free ASK1 to form homo-oligomerisation for JNK activation (Gotoh and Cooper, 1998, Liu et al., 2000, Saitoh et al., 1998). The role of ASK1 in TNF-α mediated persistent JNK signalling is also confirmed, as mouse embryonic fibroblasts derived from $ASK1^{-/-}$ mice demonstrate a rapid decline of JNK activity to near baseline by an hour, compared to continuous JNK activation even at 2 hours for $ASK^{+/+}$ (Tobiume et al., 2001). In conclusion, TNF-α activates JNK signalling via different mechanisms, for different cell types and conditions, as summarised in Figure 1.4.

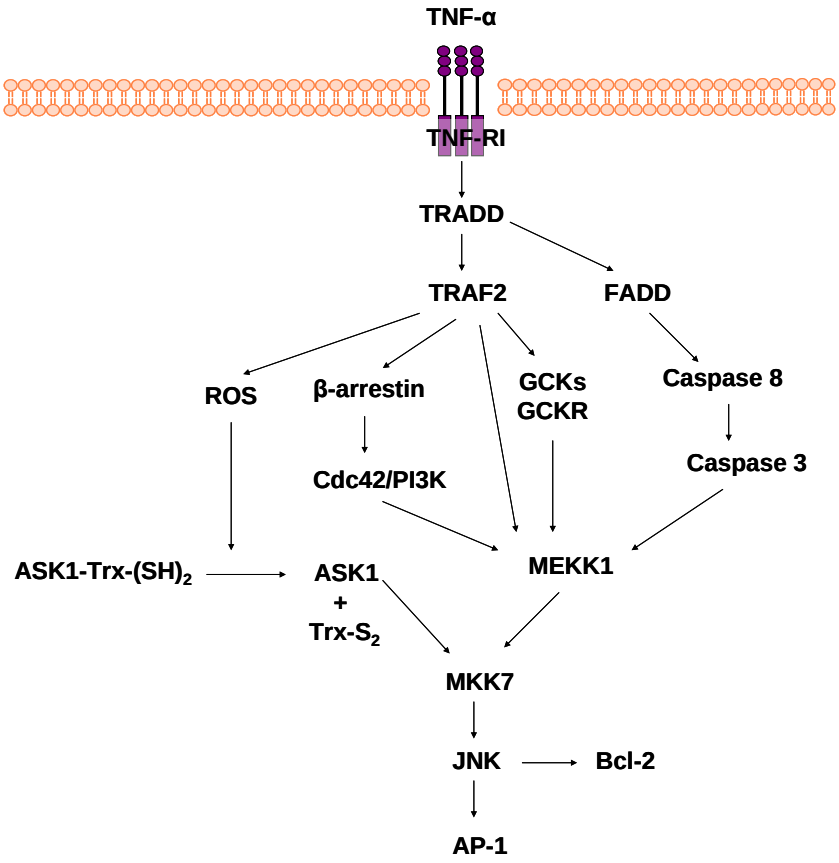


Figure 1.4: Activation of JNK signalling by TNF-α

The signalling components involved in the activation of JNK signalling by TNF-α are summarised in the diagram above (Kawamata et al., 2007, Wajant et al., 2003). TNF-α: Tumour necrosis factor-α; TNFR1: TNF receptor 1; TRADD: TNFR1-associated death domain protein; TRAF2: TNFR1 associated factor-2; FADD: Fas-associated death domain protein; ROS: Reactive oxygen species; Trx: Thioredoxin; ASK1: Apoptosis signal-regulating kinase 1; S: Sulphur; GCK: Geminal center kinase; GLK: GCK-like kinase; cdc42: Cell division control protein 42 homolog; PI3K: Phosphatidylinositol 3-kinase; MEKK1: Mitogen-activated protein kinase kinase kinase 1; MKK7: Mitogen-activated protein kinase kinase 7; JNK: c-Jun NH_2-terminal kinase; Bcl-2: B-cell lymphoma 2; AP-1: Activator protein 1.

1.4.1.1.2 Activation of other signalling pathways by TNF-α

In addition to JNK, TNF-α can also activate the other two members of the MAPK family, namely ERK and p38 MAPK. For ERK, direct interaction of TNFR1 cytoplasmic death domain with MAPK kinase activating-death domain (MADD) promotes the RIP2 recruitment to TNFR1, leading to phosphorylation of ERK2 and the AP-1 transcription factor, leading to cell proliferation (Navas et al., 1999, Schievella et al., 1997). Alternatively, TNFR1 is found to bind to tyrosine kinase receptor adaptor protein, Grb2, allowing the interaction between TNFR1 and son of sevenless (SOS) (Hildt and Oess, 1999). SOS subsequently activates Ras, c-Raf-1 and ERK signalling in HEK 293 cells (Hildt and Oess, 1999). Nevertheless concurrent activation of neutral sphingomyelinase (N-SMase) via the interaction of TNFR1 and adaptor protein FAN (factor associated with neutral sphingomyelinase) is required for the complete magnitude of ERK activation by TNF-α (Hildt and Oess, 1999). On the other hand, the exact downstream signalling components that mediate TNFR activation of p38 MAPK is less well understood. Studies on TNFR1 activation of p38 MAPK, demonstrated common upstream signalling components, as seen with activation of JNK, namely binding to TRAF2 (Yuasa et al., 1998). Therefore, TRAF2 has been shown to bind to RIP, rather than GCK, to initiate downstream activation of p38 MAPK (Yuasa et al., 1998). MKK3 knock-out studies in murine embryonic fibroblasts (MEFs) has then revealed that MKK3 mediates TNF-α activation of p38 MAPK for cytokine production (IL-1α, IL-1β and IL-6) (Wysk et al., 1999).

Furthermore, TNF-α also activates the NF-κB pathway through the recruitment of TRADD, TRAF2 and RIP to the receptor (Hsu et al., 1996). TRAF2 alone is sufficient to recruit the IκB kinase (IKK) complex, but RIP is vital for the IKK activation as shown in knockout fibroblasts (Devin et al., 2000). MEKK3 then aids RIP phosphorylation of IKK for NF-κB activation (Yang et al., 2001b). PKC ζ also participates in TNF-α mediated NF-κB activation by phosphorylating IKKβ at Ser177 and Ser181 (Lallena et al., 1999). In turn, the IKK complex phosphorylates inhibitory κB -α (IκB-α) for its degradation by E3 ubiquitin ligase Skp1/Cullin-Fbox

(Hatakeyama et al., 1999). The free p50-p65 NF-κB subunit from the IκB-α complex then enters the nucleus for targeted gene transcription.

In contrast to TNF-α mediated pro-survival via NF-κB signalling, TNF-α can also trigger a series of caspase related apoptotic events. Upon TNF-α binding, TNFR1 internalises as a death-inducing signalling complex (DISC) consisting of TNFR1, TRADD, FADD and caspase 8 to cause necrosis via sphingomyelinase and cathepsin D activation or apoptosis via caspase 3 activation (Schneider-Brachert et al., 2004). Alternatively, caspase 8 cleaves Bid to allow its C terminal truncated form (tBid) to translocate to the mitochondria, leading to cytochrome c release (Luo et al., 1998). Reactive oxygen intermediates also contribute to the cytotoxic effect of TNF-α (Goossens et al., 1995). Overall, the TNF-α mediated apoptotic effect is negatively regulated by the NF-κB pathway through increased gene transcription of cellular inhibitor of apoptosis 1/2 (cIAP1/2) to inhibit caspase 8 activation (Wang et al., 1998a).

1.4.1.2 IL-1β

Another pro-inflammatory cytokine, IL-1, also potently activates MAPK, particularly JNK and p38 MAPK, and also NF-κB signalling, as demonstrated in T helper cells (Orencole and Dinarello, 1989). The IL-1 family of receptors consists of various members, including IL-1α, IL-1β, IL-1 receptor antagonist (IL-Ra) and IL-18, which bind to IL-1 receptors (Apte et al., 2006, Bird et al., 2002, Martin and Wesche, 2002). IL-1α and IL-1β can bind to IL-1 receptor type I and type II (IL-1RI and IL-1RII respectively), both the transmembrane and soluble forms, but only binding to transmembrane IL-1RI leads to downstream signalling activation. IL-1α and IL-1β produce similar biological effects but IL-1β is only secreted during inflammation, with different tissue expression to IL-1α (Hacham et al., 2000). Both IL-1α and IL-1β are secreted as 31kDa precursors, which are cleaved into their active 17kDa form, by calpain and IL-1β-converting enzyme respectively (Howard et al., 1991, Kavita and Mizel, 1995).

1.4.1.2.1 Activation of JNK pathway by IL-1β

Similar to TNF-α, binding of IL-1β to the IL-1RI induces the recruitment of numerous adaptor proteins for the formation of intermediate signalling complexes. Binding of IL-1β to the extracellular region of IL-1RI promotes the heterodimerisation with the IL-1 receptor accessory protein (IL-1RAcP) (Huang et al., 1997, Li et al., 2005). The signalling components for IL-1β share a greater similarity with the Toll-like receptor (TLR) family, that also utilises myeloid differentiation factor 88 (MyD88), IL-1 receptor associated kinase-4 (IRAK-4), IRAK-1, TGFβ-activating kinase 1 (TAK1), TAK binding protein 1/2 (TAB1/2) and TRAF6 for JNK and NF-κB signalling (Takeda and Akira, 2004). The recruitment of MyD88-Toll-interacting protein (Tollip) complex (Burns et al., 2000, Radons et al., 2002, Wesche et al., 1997) and IRAK-4 (Fraczek et al., 2008, Li et al., 2002) to the receptor forms a stable complex of IL-1β, IL-1RI, IL-1RAcP, MyD88 and IRAK-4 as shown by mass spectrometry (Brikos et al., 2007). The serine-threonine kinase IRAK-1, is also recruited to the receptor complex (Li et al., 2001), that then undergoes phosphorylation by IRAK-4 at Thr387 and autophosphorylates the ProST region (Kollewe et al., 2004).

Next, the hyperphosphorylated IRAK-1 leaves the receptor complex to bind to TRAF6 (Cao et al., 1996b), TAB2 (Takaesu et al., 2000) and the pre-associated TAK1-TAB1 complex (Shibuya et al., 1996) to form an intermediate signalling complex (Takaesu et al., 2001, Walsh et al., 2008). TAB2/3 promotes the assembly and ubiquitination-dependent activation of TRAF6 at the CUE domain (Kishida et al., 2005, Ishitani et al., 2003). Activated TRAF6, works together with ubiquitin proteins Ubc13 and Uev1A (Deng et al., 2000), to mediate the polyubiquitination of TAK1 at Lys63 (Fan et al., 2010). Finally, TAK-1 also undergoes autophosphorylation at Thr178, Thr184, Thr187 and Ser192 (Kishimoto et al., 2000, Singhirunnusorn et al., 2005, Yu et al., 2008) to activate MKK4/7 and MKK3/6 to stimulate JNK and p38 MAPK respectively (Guan et al., 1998, Yao et al., 1997). The current model for IL-1β mediated signalling is summarised in Figure 1.5.

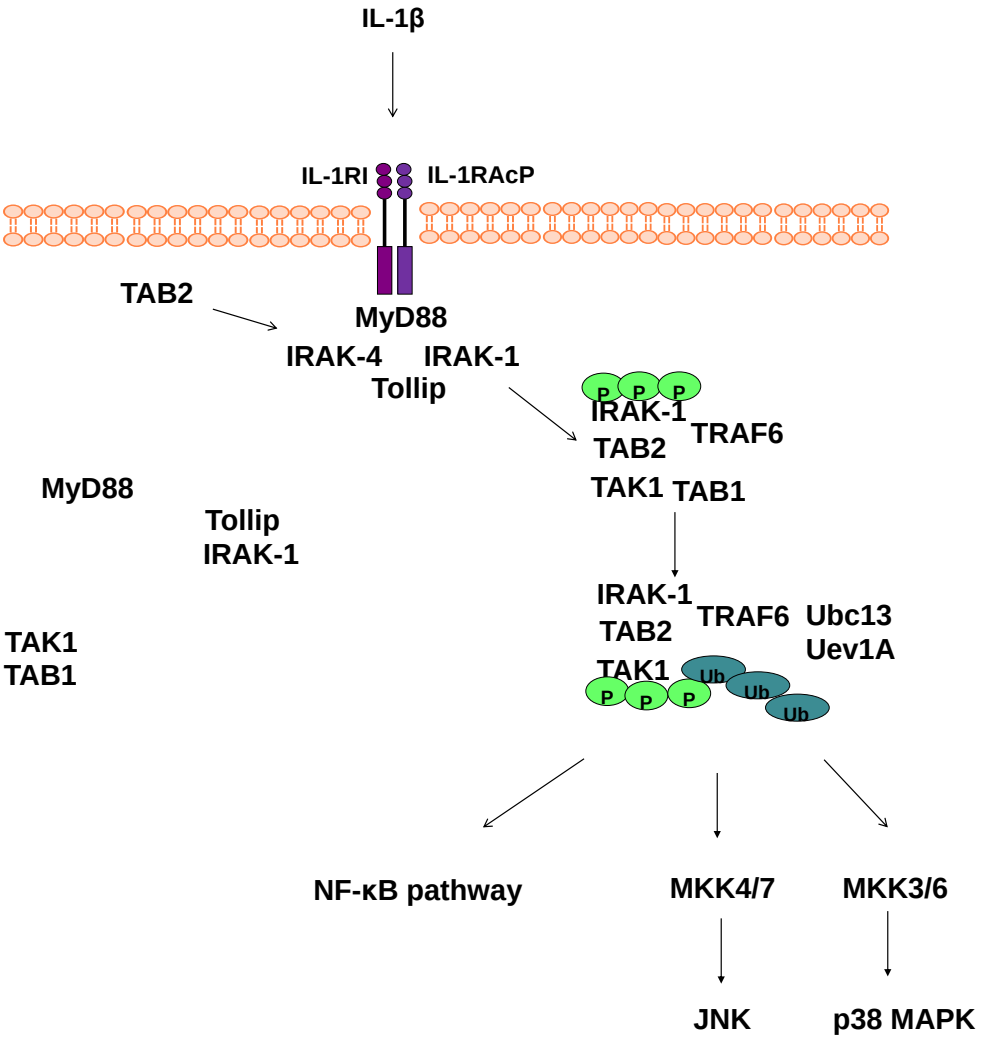


Figure 1.5: The IL-1β signalling pathway

Binding of IL-1β to its receptor IL-1RI induces the recruitment of various adaptor molecules as illustrated above (Martin and Wesche, 2002). The green P circle indicates the hyperphosphorylation whereas the blue Ub circle indicates the ubiquitination process. IL-1β: Interleukin-1β; IL-1RI: Interleukin-1 receptor type I; IL-1RAcP: Interleukin-1 receptor accessory protein; MyD88: Myeloid differentiation primary response gene 88; TAK1: TGFβ –activating kinase 1; TAB: TAK binding protein; IRAK: Interleukin-1 receptor-associated kinase; Tollip: Toll interacting protein; TRAF6: TNF receptor associated factor 6; Ubc13: ubiquitin-conjugating enzyme (E2); Uev1A: ubiquitin-like protein; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; MKK: Mitogen-activated protein kinase kinase; JNK: c-Jun N-terminal kinase.

1.4.2 UVC

Several other stress-related mediators can could also induce potent activation of JNK activity, such as UVC and certain chemotherapeutic agents (Chen et al., 1996a, Sanchez-Perez et al., 1998, Wu et al., 2002). Based on the wavelength, UV irradiation is divided into UVA (315 – 400 nM), UVB (280 – 315 nM) and UVC (100 – 280 nM) (Tyrrell, 1996). UVC irradiation has been proposed as an adjunct therapy, in combination with chemotherapy or surgery, to increase treatment efficacy. These actions are underpinned by a range of effects on different cancer cells, depending on the strength of the UVC. In tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) resistant colon carcinoma, co-treatment of TRAIL and UVC produced increased apoptosis via elevated release of cytochrome c and caspase activity (Kim et al., 2008). UVC irradiation also downregulates EGFR via p38 MAPK signalling (Yamauchi et al., 2011) and decreases the translation and accumulation of hypoxia inducible factor 1-α (HIF-1α) to reduce angiogenesis (Rapisarda and Melillo, 2007). Recent *in vivo* transplantation of fluorescence labelled lung carcinoma cells into mice has tested the implementation of UVC in cancer treatment (Kimura et al., 2010). In this study, cancer cells undergo apoptosis post UVC exposure, with a reduction in minimal residual cancer and no apparent side effects observed. The apoptotic effect of UVC has been shown to link to JNK signalling (Dunkern et al., 2001, Wu et al., 2002), thus the intermediates linking UVC to JNK signalling are described below.

1.4.2.1 Activation of the JNK pathway by UVC

Exposure to UVC consistently induces prolonged JNK activity in various cell lines (Chen et al., 1996b, Hamdi et al., 2005, Liu et al., 1995). Although the exact signalling mechanisms involved are not clearly elucidated, the studies collectively propose both the receptor and DNA damage dependent modal of JNK activation. The short term signalling is mediated by multiple membrane receptors via assemble and internalisation of TNFR1, CD95/Fas receptor and EGFR (Rosette and Karin, 1996). Downstream ligand-independent TNFR1 activation by UVC results in the recruitment of FADD to TNFR1, followed by activation of caspase 8 and 10 to

induce apoptosis (Sheikh et al., 1998). UVC also stimulates ligand-independent Fas/CD95 receptor activation, leading to binding of death-associated protein 6 (Daxx) for downstream JNK signalling and apoptosis (Wu et al., 2002). Lastly, UVC phosphorylates EGFR at Tyr1068 (Sachsenmaier et al., 1994, Cao et al., 2008), to stimulate ERK, p38 MAPK, JNK, NF-κB, and PI3K signalling (Cao et al., 2008, El-Abaseri et al., 2005).

In contrast, long term JNK signalling in response to UVC is dependent on the formation of DNA lesions, consisting of cyclobutane pyrimidine dimer (CPD) and 6-4 photoproduct (6-4 PP) (Dunkern et al., 2001, El-Mahdy et al., 2000, Ford and Hanawalt, 1997). Following enucleation, the removal of damaged DNA, JNK activity is reduced (Adler et al., 1995a), whereas deficiency in transcription-coupled repair significantly increases UVC mediated JNK signalling and apoptosis (Hamdi et al., 2005). Dunkern *et al* also reported that activation of Bcl-2, caspase 8, 9, 3 and PARP are involved in the UVC mediated apoptotic effect (Dunkern et al., 2001). The signalling mechanisms mentioned in the text are illustrated in Figure 1.6.

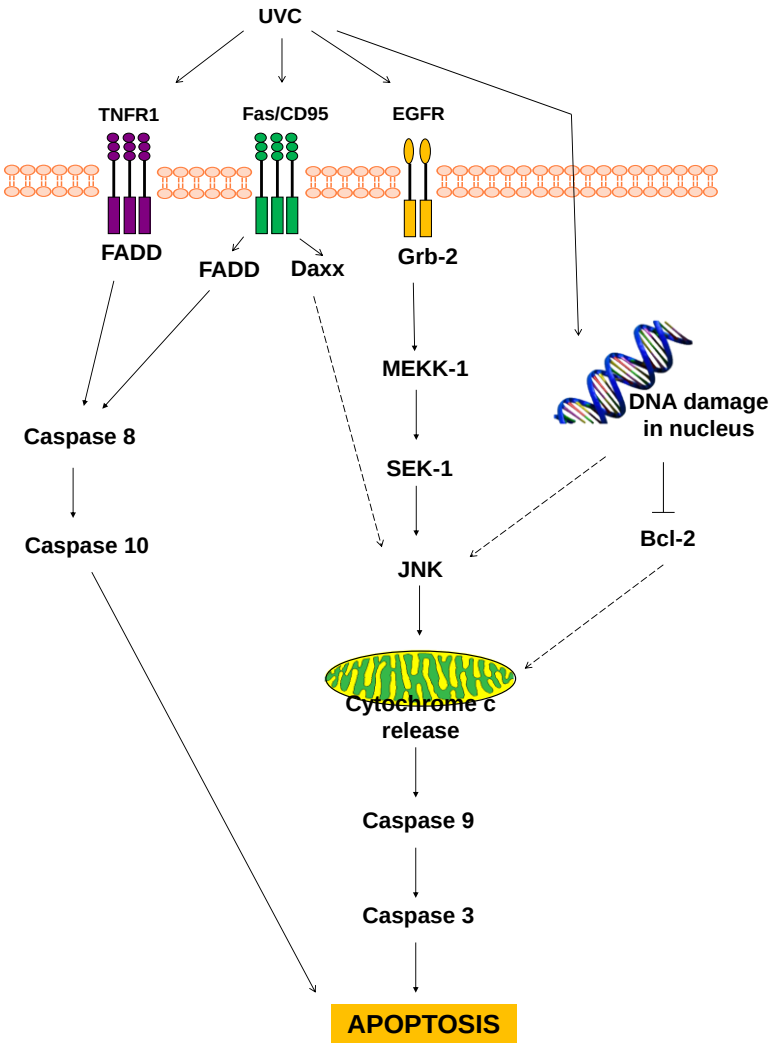


Figure 1.6: The JNK signalling pathway induced by UVC

As mentioned in the text, UVC induces JNK signalling via ligand-independent activation of surface membrane receptors and DNA damage in the nucleus to induce cell apoptosis. UVC: Ultraviolet subtype C; TNFR1: Tumour-necrosis factor receptor-1 ; FADD: Fas-associated protein with death domain; Daxx; Death-associated protein 6 ; EGFR: Epidermal growth factor receptor; Grb-2: Growth factor receptor-bound protein 2; MEKK-1: Mitogen-activated protein kinase kinase kinase 1; SEK-1: Mitogen-activated protein kinase kinase 4 (also known as MKK4); JNK: c-Jun N-terminal kinase; Bcl-2: B-cell lymphoma 2.

1.5 G-protein coupled receptors (GPCRs)

A key group of receptors, which have a diverse number of molecular functions in cells, are G-protein coupled receptors (GPCRs). These receptors have the potential to interact with and modify cytokine mediated signalling to regulate inflammation. Therefore, the characteristics and activation of GPCRs are examined below.

1.5.1 General characteristics of GPCRs

Since the cloning of bovine rhodopsin, GPCRs have been studied extensively as attractive therapeutic targets in the treatment of cancers and inflammation-related diseases (Nathans and Hogness, 1983, Osmond et al., 2010). Over 200 families and subfamilies of GPCRs have been identified through genetic studies, some remain defined as orphan receptors as their function and signalling characteristics are still unknown (Alexander et al., 2008). GPCRs are divided into several families based on the agonist binding sites on the receptor: class A rhodopsin-like, class B secretin-like, class C metabotropic glutamate/pheromone, vomeronasal and taste receptors (Vroling et al., 2011). The recent resolution of the crystal structures of GPCRs, such as A_{2A} adenosine receptor, H_1 histamine receptor, S1P1 sphingosine 1-phosphate receptor 1 and the β_1 and β_2 adrenoceptors, has allowed further understanding of the binding sites for both agonists and antagonists and other effector molecules (Rasmussen et al., 2011, Hanson et al., 2012, Warne et al., 2008, Hino et al., 2012, Shimamura et al., 2011). In general, GPCRs comprise of an extracellular N-terminus, seven α-helices, transmembrane domain and an intracellular C-terminal. Binding of an agonist at the N-terminus and/or the transmembrane domain induces conformational changes in the receptor structure to allow the receptor to interact with the coupling G proteins and effector molecules, initiating downstream signal transduction. Details on the activating mechanisms of G proteins will be discussed in the following subsections.

1.5.2 Downstream signalling pathways of heteromeric G-proteins

In general, the activation model for GPCRs involves a complex interaction among receptor, G proteins, effector molecules, regulators of G protein signalling (RGS proteins) and other accessory proteins (Offermanns, 2003). Receptor activation leads to the recruitment of trimeric Gα/Gβ/Gγ complex (Blank et al., 1991) at the C-terminal. Increase in guanosine diphosphate (GDP) – guanosine triphosphate (GTP) exchange on the Gα subunit stimulates the dissociation of Gβ/Gγ from the complex to allow Gα and Gβ/Gγ to interact with their respective effector molecules (Hepler and Gilman, 1992). The activation of G proteins is terminated by the activity of GTPase that converts GTP of Gα back to GDP, reforming the trimeric G protein complex. The cycle of GPCR activation is illustrated in Figure 1.7. RGS proteins can modulate the receptor signalling by increasing the GTPase rate of Gα (Hollinger and Hepler, 2002).

Signalling specificity of GPCRs is to an extent determined by the subgroup(s) of G proteins activated, namely $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Conklin and Bourne, 1993, Clapham and Neer, 1993). Early screening with bacteria toxins *Vibrio cholera* and *Bordetella pertussis* have led to the categorization of Gα proteins based on their sensitivity towards the toxins (Gill and Meren, 1978, Katada and Ui, 1982). Cholera toxin catalyses ADP-ribosylation at the arginine residue of $G\alpha_s$ to inhibit GTPase, leading to persistent $G\alpha_s$ activation. With the same mechanism, pertussis toxin induces ADP-ribosylation at the cysteine residue of $G\alpha_{i/o}$ to prevent the coupling of $G\alpha_{i/o}$ to GPCRs, hence inhibiting the signal transduction of $G\alpha_{i/o}$ (Mangmool and Kurose, 2011). $G\alpha_{q/11}$ and $G\alpha_{12/13}$ are insensitive to both the toxins. The downstream pathways that are linked to these G proteins are discussed in the following subsections.

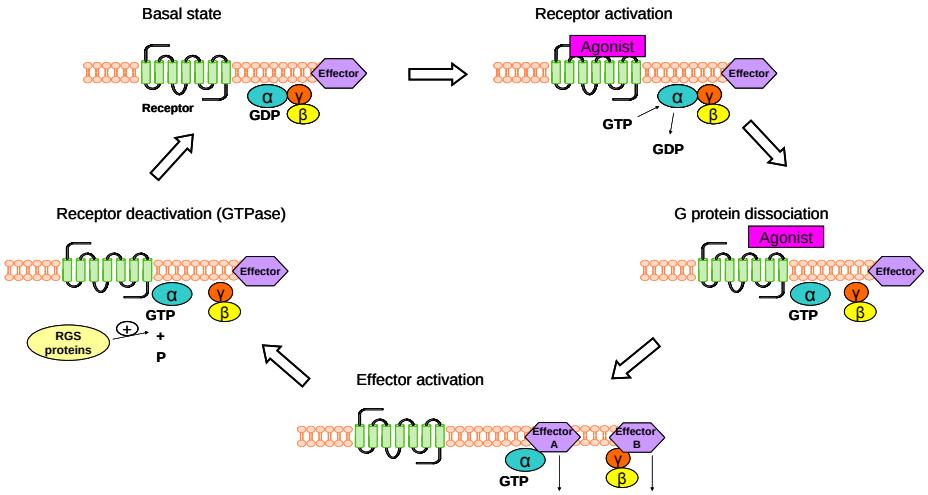


Figure 1.7: Heteromeric G protein activation by GPCRs

Activation of G proteins involves the recycling of GDP-GTP that mediates the dissociation and association of trimeric Gα/Gβ/Gγ complex. Only free Gα and Gβ/Gγ subunits can interact with the effectors for downstream signalling activation (Offermanns, 2003). GDP: guanosine diphosphate; GTP: guanosine triphosphate.

1.5.2.1 Gα dependent signalling pathways

Activation of $G\alpha_s$ is measured by an increased activity of membrane glycoprotein adenylyl cyclase (AC) (Pfeuffer et al., 1991, Gill and Meren, 1978) through direct binding of $G\alpha_s$ to the cytosolic domain of AC as demonstrated in crystallisation and binding assays (Sunahara et al., 1997, Tesmer et al., 1997). This leads to raised intracellular cyclic adenosine monophosphate (cAMP) formation, which then promotes the activation of protein kinase A (PKA) (Uhler et al., 1986a, Uhler et al., 1986b) and PKA independent exchange protein directly activated by cAMP (EPAC) (de Rooij et al., 1998, Kawasaki et al., 1998).

PKA can bind to its downstream signalling targets, including cAMP responsive element binding protein (CREB) (Montminy and Bilezikjian, 1987, Montminy et al., 1986, Yamamoto et al., 1988). On the other hand, EPAC can activate GTPase Rap1, Rap2 and ryanodine receptors to stimulate MAPK activation and increase

intracellular Ca^{2+} concentrations (de Rooij et al., 1998, Kawasaki et al., 1998, Holz et al., 2006). PKA and EPAC signalling can either work synergistically or independently in different cell systems, reflecting the diverse biological outcomes mediated by G_s activation which are yet to be understood completely. For instance, both PKA and EPAC synergises to increase phophodiesterase activity (Dodge-Kafka et al., 2005) and neurotensin secretion via increased Rap1 activity (Li et al., 2007), but exert opposing regulation on the PKB/Akt pathway (Mei et al., 2002). Signalling pathways downstream of $G\alpha_s$ are illustrated in Figure 1.8.

In contrast to $G\alpha_s$, the binding of $G\alpha_{i/o}$ to AC (Dessauer et al., 1998) reduces the activity of AC. Thus, $G\alpha_{i/o}$ acts as a negative regulator of $G\alpha_s$, to limit the increase in intracellular cAMP. Activation of $G\alpha_{i/o}$ allows the release of free $G\beta/\gamma$ to activate both PI3K and MAPK (Galve-Roperh et al., 2002, Rueda et al., 2000). Another recent study reveals that $G\alpha_{i/o}$ can modulate cAMP and ERK activity via binding to the regulatory subunit for PKA, which plays a pivotal role in $G\alpha_s$-coupled signalling (Stefan et al., 2011). For instance, the $G\alpha_{i/o}$-coupled cannabinoid receptor (CB_1) activates ERK via PI3K (Galve-Roperh et al., 2002).

Following these initial discoveries, PCR revealed the presence of a third $G\alpha$ protein, $G\alpha_{q/11}$, sensitive to neither pertussis nor cholera toxin (Strathmann and Simon, 1990). Activation of $G\alpha_{q/11}$ leads to initiation of inositol phosphate signalling via phosphoinositide-specific phospholipase C ($PLC\beta$) activation (Blank et al., 1991). In the plasma membrane, $PLC\beta$ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Berridge et al., 1983, Berridge, 1987). IP_3 then stimulates IP_3 receptors to release intracellular Ca^{2+} mainly from the endoplasmic reticulum (Burgess et al., 1984, Nahorski and Potter, 1989) whereas DAG activates the protein kinase C (PKC) family of enzymes (Ganong et al., 1986). Recent crystallisation and yeast two hybrid screening techniques have led to the identification of new signalling effectors of $G\alpha_q$, namely Ras-homology protein (RhoA) and Ric-8 respectively (Lutz

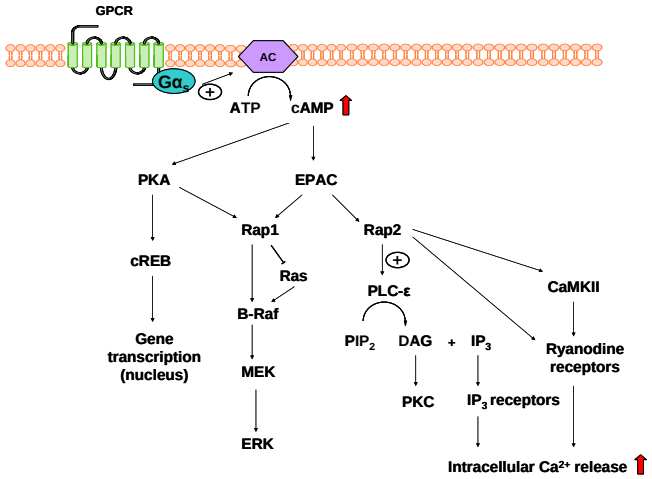
et al., 2007, Nishimura et al., 2006). For instance, knockdown of guanine exchange factor Ric-8A reduces $G\alpha_q$ dependent ERK activation and intracellular Ca^{2+} mobilisation, suggesting that Ric-8A is required for the potentiation of $G\alpha_q$ signalling activation (Nishimura et al., 2006). Scaffolding proteins caveolin and flotilins also aid in the signal transduction of $G\alpha_q$ by forming lipid rafts to segregate the signalling components (Bhatnagar et al., 2004, Sugawara et al., 2007). The downstream signalling of $G\alpha_{q/11}$ is illustrated in Figure 1.8.

A fourth class of Gα proteins, $G\alpha_{12/13}$, was later cloned by Strathmann and Simon (Strathmann and Simon, 1991). Unlike the other Gα proteins discussed above, $G\alpha_{12/13}$ does not couple to the conventional cAMP, Ca^{2+} or IP_3 signalling cascades as these levels remain unchanged upon selective $G\alpha_{12/13}$ activation. Expressed ubiquitously, $G\alpha_{12/13}$ plays an important role in cell proliferation through its interaction with Rho (Siehler, 2009). Furthermore, $G\alpha_{12/13}$ mediates the angiotensin II dependent ROS production to stimulate JNK and p38 MAPK signalling in rat cardiac myocytes (Nishida et al., 2005).

1.5.2.2 Gβγ dependent signalling pathways

In relation to Gβγ subunits, an initial model of GPCR activation implicated a singular role for Gβγ as a regulator of Gα at the membrane, increasing the affinity of Gα towards GDP to stabilise the inactive form by 100-fold (Higashijima et al., 1987). This hypothesis has been disputed by the discovery of Gβγ mediated activation of cardiac potassium channels stimulated via the muscarinic receptor (Logothetis et al., 1987). Various isoforms of $G\beta_{1\text{-}5}$ and $G\gamma_{1\text{-}14,\ rod,\ cone}$ proteins have since been cloned (Offermanns, 2003, Smrcka, 2008) which reveals a wider role for Gβγ subunits in GPCR signalling. For example, purified PLCβ2 binds directly to Gβγ at amino acid residues 574-576 to mediate PLCβ activation (Lehmann et al., 2007).

A



B

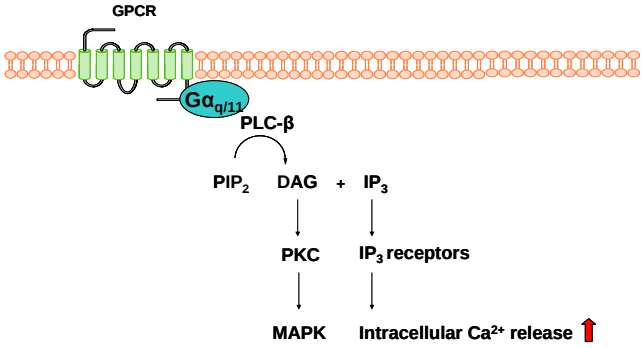


Figure 1.8: $G\alpha_s$ and $G\alpha_{q/11}$ dependent signalling

Activation of $G\alpha_s$ leads to downstream activation of AC, PKA and EPAC to increase intracellular ERK and Ca^{2+} concentration in (A). Activation of $G\alpha_{q/11}$ leads to formation of DAG and IP_3 which in turn activates a cascade of downstream signalling activation including MAPK and intracellular Ca^{2+} release in (B). Other non-Rap dependent signalling by EPAC or RhoA and Ric-8 for $G\alpha_q$ is not illustrated in the diagram. ATP: Adenosine-5'-triphosphate; cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; EPAC: Exchange protein directly activated by cAMP; cREB: cAMP response element-binding; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal regulated kinases ; PLC: Phospholipase C ; PIP_2: Phosphatidylinositol 4,5-bisphosphate; DAG: Diacylglycerol; PKC: Protein kinase C; IP_3: Inositol trisphosphate; CaMKII: Ca^{2+}/calmodulin-dependent protein kinases II; Ca^{2+}: Calcium ion; MAPK: Mitogen-activated protein kinase.

1.5.3 Downstream signalling pathways of monomeric G-proteins

Small GTP binding proteins, or monomeric G-protein, have been implicated in mediating the effects of GPCRs. Small molecular weight G proteins are divided into at least five subfamilies: Ras, Rho/Rac/cdc42, Rab, Sar1/Arf and Ran (Takai et al., 2001). The widely studied Ras, is originally discovered as an oncogene for sarcoma virus (Chien et al., 1979), that localises Raf-1 to the plasma membrane (Leevers et al., 1994, Vojtek et al., 1993) for downstream MEK and ERK signalling pathway activation (Minden et al., 1994a). Studies have also demonstrated that Ras and RhoA bind to distinct sites on PLCε to stimulate downstream PIP_2 hydrolysis and IP_3 formation (Seifert et al., 2008, Song et al., 2001a). MAPK activation is also linked to the Rho/Rac/cdc42 subfamily as overexpression of constitutively active Rac and cdc42 results in increased JNK and p38 MAPK activity (Coso et al., 1995b, Minden et al., 1995). Rab, Sar1/Arf and Ran are involved in the intracellular trafficking mechanisms (Takai et al., 2001), for example, Rab11a mediates proteinase activated receptor 2 (PAR_2) trafficking to the cell surface, that requires prior receptor palmitoylation in the Golgi apparatus (Adams et al., 2011). Ran is also found to increase secreted modular calcium-binding protein-2 (SMOC-2) expression to stimulate fibroblast growth through ERK and JNK signalling (Milano et al., 2012).

More recently, it has been shown that monomeric G proteins can also modulate the activity of GPCRs. For instance, p63RhoGEF, acts as a signalling intermediate, and together with RhoA, binds to $G\alpha_q$ to increase downstream RhoA dependent outcomes, such as actin stress fibre formation, cell rounding and serum response factor dependent gene expression (Lutz et al., 2007). Studies have also found that Arf1 directly binds to muscarinic M_3 and 5-hydroxytryptamine 2A ($5\text{-}HT_{2A}$) receptor to activate the downstream phospholipase D (PLD) pathway (Johnson et al., 2006, Mitchell et al., 2003). Thus, Arf1 could work co-operatively with PKC to activate PLD signalling, as shown for cholecystokinin octapeptide (CCK) receptor activation in intestinal smooth muscle cells (Murthy et al., 2001). In conclusion, GPCRs, depending on the receptor subtype and the expression of G proteins (both

heteromeric and monomeric) in specific cell types, can produce downstream signal transduction via multiple mechanisms.

1.5.4 Non-G protein dependent signalling of GPCRs

In addition to G-proteins, GPCRs can also interact with other signalling molecules for downstream signalling and receptor trafficking. Amongst the signalling molecules, the interaction between GPCRs, β-arrestins and clathrin is most well understood as a scaffolding platform to co-localise GPCR and other signalling components (Defea, 2008). Several GPCRs, including PAR_2 and the β_2-adrenoceptor, can couple to β-arrestin to mediate receptor endocytosis, subsequently induce the formation of a complex with Raf-1 and activated ERK, to prolong ERK signalling (DeFea et al., 2000, Shenoy et al., 2006). GPCRs can also activate the ERK pathway through transactivation of tyrosine kinase receptors, most notably the EGFR, via metalloproteinase mediated release of heparin-bound EGF (Liebmann, 2011, Prenzel et al., 1999). Recent studies have revealed a wide range of molecules and receptors that interact with GPCRs. For example, immunoprecipitation experiments have demonstrated that $P2Y_{12}$ interacts with Na^+/H^+ exchanger regulatory factor (NHERF1) to potentiate receptor internalisation (Nisar et al., 2012). A complex between β2 aderenoceptor and hyperpolarization-activated and cyclic nucleotide-gated channel 4 (HCN4) has also been identified that underpins the regulation of cardiac ion channels following β-adrenoceptor activation (Greene et al., 2012).

1.5.5 GPCR mediated regulation of JNK signalling

Various studies have demonstrated that different GPCRs that couple to $G\alpha_q$, $G\alpha_s$ and $G\alpha_{i/o}$ can activate JNK signalling in different cell systems, such as the angiotensin II type1 receptor (AT_1) in VSMCs, the dopamine (D_1) receptor in epithelial cells, the muscarinic receptor 1 (M_1) in fibroblasts and the endothelin-1 (ET-1) receptor in neural progenitor cells (Eguchi et al., 2001, Mizuno et al., 2005, Chan and Wong, 2005, Coso et al., 1995a). Signalling intermediates including Src family tyrosine kinases, Rho, Rac1, cdc42 and cAMP are involved in downstream JNK activation by

the various G proteins (Yamauchi et al., 2001b, Yamauchi et al., 2001a, Teramoto et al., 1996). However, it should be noted that the magnitude of JNK activity is generally low (1 – 2 fold stimulation) and transient (reduced to baseline within 30 – 60 minutes), and is detected following over-expression of receptors in transfected cell systems or using high concentrations of agonists (Chan and Wong, 2004, Chan and Wong, 2005, Mizuno et al., 2005). Thus, the physiological role of JNK activation by GPCRs remains largely unknown.

1.5.6 A potential negative regulator of JNK signalling by GPCRs

Various studies have demonstrated that activation of GPCRs and/or their downstream signalling molecules can interact with the signalling activity of cytokines. Studies from the lab have clearly shown that activation of PAR_2 and $P2Y_2$ receptors inhibit TNF-α stimulated JNK signalling (McIntosh et al., 2010, Paul et al., 2000). Other laboratories have also confirmed this regulatory effect of GPCR activation in cytokine mediated signalling, as the histamine receptor-1 (H_1) and cannabinoid receptor-2 (CB_2) can also reduce TNF-α induced MAPK activity (Rajesh et al., 2008, Steffel et al., 2006). These results reveal an interesting hypothesis that irrespective of the subtypes of G-protein linked to the GPCR, GPCRs have the ability to inhibit cytokine induce downstream signalling, particularly JNK. Furthermore, selective inhibition of G-protein activity in these studies reversed the inhibitory effect of GPCRs. For instance, inhibition of $G\alpha_{q/11}$ with the pharmacological inhibitor YM254890 prevented PAR_2 mediated inhibition of TNF-α induced JNK activity (McIntosh et al., 2010).

In addition, activation of downstream GPCR signalling components also inhibited cytokine induced signalling in a similar manner as observed with activation of GPCRs. Direct activation of PKC, with phorbol 12-myristate 13-acetate (PMA), prevented the formation of TRADD, RIP, TRAF2 and TNF-R1 receptor complex that in turn inhibited TNF-α mediated JNK, NF-κB signalling and ROS production (Byun et al., 2006). Furthermore, stimulation of $G\alpha_s$ downstream signalling with

forskolin and cicaprost to increase intracellular cAMP, inhibits the surface expression of ICAM-1 and VCAM-1 induced by TNF-α, IL-1α and IL-1β in HUVECs, human vascular and airway smooth muscle cells (Ghersa et al., 1994, Panettieri et al., 1995). In monocytes, an increase in cAMP via $P2Y_{11}$ receptor activation also inhibits the pro-inflammatory secretion of TNF-α and MCP-1 by TLR2, TLR4 or TLR2/6 (Kaufmann et al., 2005). Taken together these studies suggest that GPCR mediated inhibition of inflammatory MAPK signalling may be a bonafide effect. Therefore it is important to understand the pharmacological characteristics and coupling of these receptors to intracellular signalling pathways. This is discussed below.

1.5.7 Purinoceptors

The physiological roles of purines and pyrimidines have been studied for over five decades. An early study injecting the bullock heart muscle extract into guinea pigs demonstrated that adenine was present in the heart and could induce changes in electroradiographic recordings (Drury and Szent-Gyorgyi, 1929). Then the importance of purine adenosine-5'-triphosphate (ATP) as an intracellular energy source in driving glucose oxidation was revealed by measuring the contraction of insect flight muscle (Sacktor, 1955). However it was not until Burnstock and colleagues revealed the ability of ATP to function as a neurotransmitter in gut and urinary bladder (Burnstock 1972), that the concept of purinoceptor activation was considered (Burnstock, 1972, Ralevic and Burnstock, 1998, Abbracchio et al., 2006).

Based on agonist potency orders, purinoceptors are divided into adenosine receptors (P1) that are activated by adenosine; and purinergic receptors (P2) that are activated by ATP, UTP, ADP, UDP and UDP-glucose (Ralevic and Burnstock, 1998). P1 receptors are further classified into adenosine A_1, A_{2A}, A_{2B} and A_3 receptors. On the other hand, P2 receptors are divided into P2X and P2Y receptor subfamilies based on their structural homology and pharmacological properties (Fredholm et al., 1994). To date, seven ionotropic ligand-gated ion channels of P2X receptors ($P2X_{1-7}$) (Le et al.,

1997, Valera et al., 1994, Urano et al., 1997, Garcia-Guzman et al., 1997(b), Lynch et al., 1999, Rassendren et al., 1997) and eight metabotropic P2Y isoforms ($P2Y_{1, 2, 4, 6, 11, 12, 13 \text{ and } 14}$) have been cloned in humans (Ayyanathan et al., 1996, Chambers et al., 2000, Communi et al., 1995, Communi et al., 1997, Communi et al., 1996, Communi et al., 2001, Hollopeter et al., 2001, Nguyen et al., 1995).

Under physiological and disease conditions, these class A rhodopsin-like purinoceptors are activated due to high extracellular ATP concentrations as detected in hypoxia (Bodin and Burnstock, 1995) and inflammatory conditions (Bodin and Burnstock, 1998). In the presence of various ectonucleotidases, ATP has a short half-life and is prone to undergo degradation to ADP, AMP and adenosine (summarised in Figure 1.9) (Goding et al., 2003). Endothelial and cancer cells express abundant ecto-nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and ecto-5'-nucleotidase compared to other cell types (Dzhandzhugazyan et al., 1998, Mandapathil et al., 2009, Wood et al., 2002). Shear stress also induces the release of soluble ecto-ATPase along with ATP secretion (Yegutkin et al., 2000), and in sympathetic nerve terminals, ATP can be released from the same vesicle as the equivalent ecto-ATPase (Todorov et al., 1997). The activity of ectonucleotidases contributes to the increase in the local concentration of ATP and their metabolites on the endothelial cell surface and the accumulation of adenosine in the hypoxic tumour core (Ohta et al., 2006, Wood et al., 2002). The general characteristics and downstream signalling of purinoceptors are described as follows.

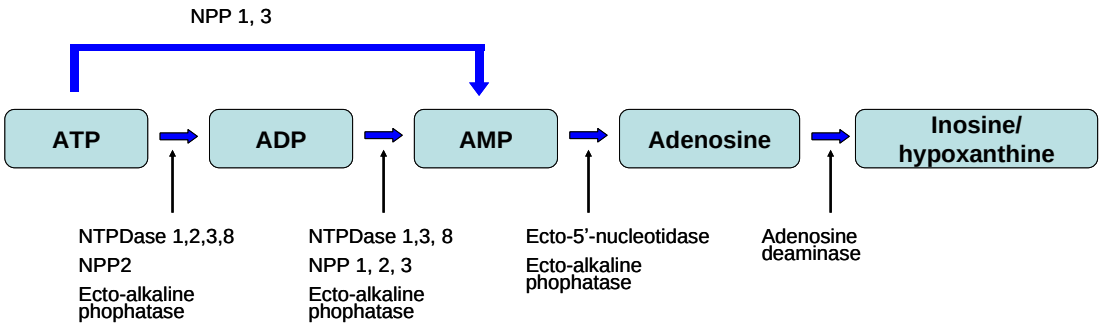


Figure 1.9: The metabolism of ATP by various ectonucleotidases

ATP undergoes a stepwise enzymatic degradation to ultimately form inosine/hypoxanthine. Amongst these ectonucleotidases, NTPDase 1 and ecto-5'-nucleotidase are highly expressed in endothelial and cancer cells (Dzhandzhugazyan et al., 1998, Mandapathil et al., 2009, Wood et al., 2002, Knowles and Li, 2006). ATP: Adenosine-5'-triphosphate; ADP: Adenosine diphosphate; AMP: Adenosine monophosphate; NPP: Nucleotide pyrophosphatase/phosphodiesterase; NTPDase: Ecto-nucleoside triphosphate diphosphohydrolases.

1.5.7.1 Purinergic P2Y receptors

In human, eight P2Y isoforms are expressed, namely $P2Y_{1,}$ $P2Y_{2,}$ $P2Y_{4,}$ $P2Y_{6,}$ $P2Y_{11,}$ $P2Y_{12,}$ $P2Y_{13}$ and $P2Y_{14}$ that confer different binding affinities towards ATP, ADP, UTP and UDP (summarised in Table 1.1) (Ayyanathan et al., 1996, Chambers et al., 2000, Communi et al., 1995, Communi et al., 1997, Communi et al., 1996, Communi et al., 2001, Hollopeter et al., 2001, Nguyen et al., 1995). In both endothelial and cancer cells, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors are most abundantly expressed and play a functional role in these systems (Wang et al., 2002, Ding et al., 2011a, White and Burnstock, 2006). Collectively, these receptor subtypes mainly couple to $G_{q/11}$ to initiate downstream signalling effects, as revealed by early studies that demonstrated ATP stimulated increases in IP_3 formation and intracellular Ca^{2+} concentration in endothelial cells (Forsberg et al., 1987, Pirotton et al., 1987). The following discussion will focus on the downstream signalling of $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors and their roles in atherosclerosis and cancer.

Table 1.1: Summary of purinergic receptors subtypes expressed in humans (Alexander et al., 2011) Only the endogenously available agonists for human are stated in the table below.

Purinergic receptors subtype	G-protein coupling & signalling transduction mechanism	Potency ranking for agonists
$P2Y_1$	$G_{q/11}$	ADP > ATP
$P2Y_2$	$G_{q/11}$	UTP ≈ ATP
$P2Y_4$	$G_{q/11}$	UTP > ATP
$P2Y_6$	$G_{q/11}$	UDP >> UTP > ATP
$P2Y_{11}$	$G_{q/11}$ and G_s	ATP > UTP
$P2Y_{12}$	$G_{i/o}$	ADP >> ATP
$P2Y_{13}$	$G_{i/o}$	ADP >> ATP
$P2Y_{14}$	$G_{q/11}$	UDP-glucose

1.5.7.1.1 Signalling activation of $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors

$P2Y_1$: The first P2Y receptor cloned, $P2Y_1$ receptor, is activated by both ADP and ATP. These nucleotides have full and partial agonist activity as shown by [γ^{32}P]GTP hydrolysis (Filtz et al., 1994, Waldo and Harden, 2004). Coupling of $P2Y_1$ receptor with $G_{q/11}$ links to downstream phospholipase C and a rapid increase in Ca^{2+} signalling (Filtz et al., 1994, Palmer et al., 1998), that leads to downstream effects such as exocytosis, contraction and proliferation (Berridge, 2012). In the context of the endothelium, this results in an increase in nitric oxide release, to mediate vasodilation (see section 1.5.7.1.2). Activation of $P2Y_1$ receptor also initiates PI3K signalling and transactivation of tyrosine kinase receptors, both of which function to dampen $P2Y_1$ receptor mediated ERK activation (Sellers et al., 2001, Shen and DiCorleto, 2008). Furthermore, in HUVECs, ADP stimulation of $P2Y_1$ receptor also results in the phosphorylation of p38 MAPK and JNK which in turn leads to the activation of p90rsk, c-jun and transcription factor-2 (Shen and DiCorleto, 2008).

$P2Y_2$: Amongst P2Y subfamily members, the signalling mechanisms mediated through $P2Y_2$ receptor (formerly known as P2U) has been studied most extensively. $P2Y_2$ receptor couples classically to $G_{q/11}$ to initiate downstream activation of IP_3, DAG, PKC and intracellular Ca^{2+} release (Enomoto et al., 1996). However activation of $P2Y_2$ receptor has also been shown to couple to $G_{i/o}$ proteins (Bagchi et al., 2005), and to activate integrins (Bagchi et al., 2005), PI3K (Wilden et al., 1998) and MAPK (Tu et al., 2000, Wilden et al., 1998, Thevananther et al., 2004) signalling. MAPK signalling mediated by $P2Y_2$ receptor is also linked to the mechanisms of receptor desensitisation as siRNA of G-protein coupled receptor kinase 2 (GRK2) and β-arrestin 2 that prevent $P2Y_2$ receptor desensitisation results in persistent ERK and p38 MAPK activity in rat aortic smooth muscle cells (Morris et al., 2012).

Although MAPK pathway activation has been implicated in the physiological and the pathophysiological effects of $P2Y_2$ receptor, direct evidence regarding its signalling mechanisms are limited. Studies have mainly focused on the multiple models of ERK activation through $P2Y_2$ receptor stimulation by ATP or UTP, as observed in various cell lines, such as osteoblasts, colon and breast cancer cells (Katz et al., 2006, Buzzi et al., 2009, Bilbao et al., 2010). Based on the finding that ATP and UTP activation of ERK is ablated in the presence of pertussis toxin, it is implied that $P2Y_2$ receptor couples to $G_{i/o}$ for ERK signal transduction (Soltoff et al., 1998). Downregulation of PKC also did not abolish the ERK signal completely, suggesting that both PKC dependent and independent pathways are important in $P2Y_2$-ERK signalling (Soltoff et al., 1998). The PKC-independent pathway involves phosphorylation of related adhesion focal tyrosine kinase (RAFTK) which subsequently transactivates EGFR for ERK activation (Soltoff, 1998). In contrast, a recent study in keratinocytes has shown that UTP activates $P2Y_2$ receptor to inhibit EGF stimulation of Raf and ERK activity (Faure et al., 2012), suggesting that there could be a cell dependent signalling mechanism for $P2Y_2$ receptor. Other studies also show that $P2Y_2$ receptor stimulation leads to p38 MAPK activation in rat renal mesengial, osteoblasts, breast and colon cancer cells (Bilbao et al., 2010, Buzzi et al., 2009, Huwiler et al., 2000, Katz et al., 2006), whereas phosphorylation of JNK, c-Jun and c-Fos are observed in

hepatocytes (Thevananther et al., 2004). Nevertheless the exact upstream signalling components involved in p38 MAPK and JNK signalling are not directly shown or suggested in these studies.

$P2Y_{11}$: The most abundantly expressed P2Y receptor in HUVECs, $P2Y_{11}$, cloned from a human cDNA placenta library, has 33% similarity in amino acid sequence as $P2Y_1$ receptor (Communi et al., 1997). Activation of $P2Y_{11}$ receptor leads to IP_3 and cAMP accumulation demonstrating the unique bi-G protein coupling of $P2Y_{11}$ receptor to both $G_{q/11}$ and G_s (Communi et al., 1997, Communi et al., 1999). In addition to these models of G-protein coupling, recent studies have suggested the coupling of $P2Y_{11}$ receptor to G-protein independent signalling. Confocal imaging results have shown that ATP mediated activation of $P2Y_{11}$ receptor induces receptor internalisation concurrently with β-arrestin-2 translocation only in the presence of GPCR kinase, GRK2 (Hoffmann et al., 2008). Furthermore, in human embryonic kidney 293 (HEK293) and astrocytoma cells, internalisation of $P2Y_{11}$ receptor requires co-expression and co-internalisation with $P2Y_1$ receptor (Ecke et al., 2008), thus it is tempting to suggest that the overall pattern of $P2Y_{11}$ receptor signalling, such as ERK, is determined by the co-operative activity of $P2Y_1$ receptor.

1.5.7.1.2 The role of purinergic receptors in atherosclerosis

The cellular studies outlined above implicated that purinergic receptors could mediate the development of cardiovascular diseases. Early experiments examining ATP stimulation in the blood vessels proposed a protective effect of purinergic receptors in the cardiovascular system due to a rapid increase in L-arginine uptake and nitric oxide release in endothelial cells (Bogle et al., 1991). A more current study demonstrated phosphorylation of nitric oxide synthase (eNOS) at S617, S635 and S1179 by ATP in another endothelial cell line (Cale and Bird, 2006). Although the Akt/PKB pathway has been implicated in the shear stress-dependent phosphorylation of eNOS, this pathway is not involved in the purinergic receptor induced eNOS phosphorylation (Dimmeler et al., 1999). Instead, in HUVECs, purinergic receptors

($P2Y_1$, $P2Y_2$, and possibly $P2Y_4$ receptor) phosphorylate eNOS in a Ca^{2+} and PKCδ dependent manner (da Silva et al., 2009). This cellular observation also correlates with a tissue vessel study of ATP-eNOS dependent vasodilatation through activation of both P2X and P2Y receptors (Liu et al., 2004a). Moreover, another study in coronary artery smooth muscle has found that ATP stimulates ERK activation through MEK to stimulate cell proliferation (Wilden et al., 1998). However due to limitations in cloning techniques (King et al., 1998), early studies on the role of ATP in the cardiovascular system could only speculate as to the P2Y subtype involved based on the potency ranking of the agonists employed. Recent advances in molecular techniques and the availability of specific receptor agonists and antagonists has allowed this to be better clarified. In the cardiovascular system, $P2Y_1$, $P2Y_2$ and $P2Y_{11}$ receptors are the main signal transducers for ATP. The roles of these receptors are discussed as below.

Early studies on $P2Y_1$ receptor concentrated on the effect of ADP in stabilising the platelet aggregation induced by thrombin via aiding fibrinogen binding to the activated platelet (Cattaneo et al., 1990, Plow and Marguerie, 1980). Later cloning has proven that $P2Y_1$ receptor is responsible for the effect of ADP in causing platelet shape change via Ca^{2+} mobilisation in the platelet (Hechler et al., 1998, Jin et al., 1998). Later mouse knockout studies demonstrate a role for $P2Y_1$ receptor in vascular inflammation. Double $P2Y_1^{-/-}$ and $ApoE^{-/-}$ knockout mice have smaller atherosclerotic lesions, lower macrophage infiltration and VCAM-1 expression in comparison with $ApoE^{-/-}$ equivalents (Hechler et al., 2008). Following this study, the same group observed a reduction in P-selectin, VCAM-1 and ICAM-1 expression in $P2Y_1$ receptor deficient endothelial cells compared to wild type (Zerr et al., 2011). This reduction in adhesion molecule expression is attributed to reduced transcription factor (ATF2) activation via inhibition of $P2Y_1$ receptor induced p38 MAPK activation (Zerr et al., 2011).

$P2Y_2$ receptor has been studied extensively as a potential therapeutic target in inflammation as IL-1β, interferon-γ and TNF-α upregulate $P2Y_2$ receptor expression in VSMCs via a PKC, cyclooxygenase and MAPKK dependent manner (Hou et al., 2000, Hou et al., 1999). Studies in VSMCs (Wilden et al., 1998) and carotid artery endothelial cells (Seye et al., 2002) also support a role for $P2Y_2$ receptor in promoting mitogenesis, hence inducing the atherosclerotic lesion formation. However, $P2Y_2$ receptor knockout mice have been shown to have salt-resistant arterial hypertension with reduced neutrophil infiltration and serum lactate dehydrogenase at 24 hours post myocardial infarction compared to wild type (Cohen et al., 2011, Rieg et al., 2007). Although a direct link with atherosclerosis has not been shown, rat models of aortic injury demonstrated high expression levels of $P2Y_2$ receptor in VSMCs of atherosclerotic lesions (Seye et al., 1997).

Due to the absence of $P2Y_{11}$ receptor in the rat and mouse genome (von Kugelgen, 2006), there is lack of *in vivo* evidence about the role of $P2Y_{11}$ receptor in cardiac disease. Genetic analysis of patients with acute myocardial infarction (AMI) reveals that Ala-87-Thr polymorphism is associated with a higher risk of AMI and plasma C reactive protein (Amisten et al., 2007). In endothelial cells, stimulation of $P2Y_{11}$ receptor with ATP inhibits cell proliferation by inducing cell cycle arrest in the S phase (Xiao et al., 2011). Collectively, these studies suggest that P2Y receptors are attractive therapeutic target in cardiovascular diseases.

1.5.7.1.3 The role of purinergic receptors in cancer

The anti-tumour effect of ATP was first tested *in vivo* via systemic or intraperitoneal infusion of ATP into mice, which resulted in inhibition of tumour growth and host weight lost (Rapaport and Fontaine, 1989, Rapaport, 1988). Other *in vitro* studies in breast, colon and pancreatic carcinoma cell lines have also supported the anti-cancer effect of ATP (Spungin and Friedberg, 1993, Rapaport, 1983). The reduction in cell proliferation, due to cell cycle arrest in the S phase, was observed only after 2-4 days with daily addition of ATP. The effects of ATP appear to be concentration related as

low (10 μM) and high doses (1 – 5 mM) produced an increase and decrease in the proliferation of the squamous cell carcinoma cell line A431 (Greig et al., 2003), suggesting that different signalling pathways or receptor subtypes could be stimulated by different levels of ATP.

The roles of $P2Y_1$ and $P2Y_2$ receptors in cancer are well studied as these receptors are most commonly expressed in patient tissue samples and established cancer cells as shown in RT-PCR (Sellers et al., 2001, Hopfner et al., 2001, Maaser et al., 2002, Hopfner et al., 1998, Buzzi et al., 2009, Wei et al., 2011). For activation of $P2Y_1$ receptor, application of a selective agonist decreases proliferation of A375 melanoma cells (White et al., 2005) and induces apoptosis in astrocytoma and prostate cancer cells as measured by caspase 3 and annexin-V activity (Sellers et al., 2001, Wei et al., 2011). In contrast, in cervical cancer Hela cells, incubation with various $P2Y_1$ agonists, including ADP and 2-MeSADP, stimulate cell proliferation as detected via [^{3}H] thymidine incorporation (Buvinic et al., 2007). This study also shows that $P2Y_1$ receptor stimulates cell growth through transactivation of EGFR with increased EGFR biosynthesis and $P2Y_1$ receptor expression.

Furthermore, activation of $P2Y_2$ receptor also produces cell dependent proliferative or anti-proliferative effects. For instance, an accumulation in S phase of the cell cycle, anti-proliferative and apoptotic effects are observed in colon and oesophageal cancer cells (Hopfner et al., 1998, Hopfner et al., 2001, Maaser et al., 2002). The anti-cancer effects relates to intracellular Ca^{2+} signalling as the calcium chelator BAPTA abolishes this effect in colon cancer cells (Hopfner et al., 2001). Conversely, the proliferative effect of $P2Y_2$ receptor is found in lung cancer, melanoma and non-melanoma skin cancer (Schafer et al., 2003, White et al., 2005, Greig et al., 2003), which is mediated through PLC, Ca^{2+}/calmodulin-dependent protein kinase II and NF-κB signalling pathways. Interestingly, stimulation of $P2Y_2$ receptor also modulates the efficacy of chemotherapeutic drugs, synergising with 5-fluorouracil

and cisplatin, but antagonising the effect of paclitaxel and etoposide (Hopfner et al., 2001, Schafer et al., 2003).

Compared to $P2Y_1$ and $P2Y_2$ receptors, there is only limited evidence regarding the role of $P2Y_{11}$ receptor in cancer. In hormone-refractory prostate cancer, $P2Y_{11}$ receptor is indicated in growth inhibition and apoptosis via a calcium independent mechanism (Shabbir et al., 2008). Activation of $P2Y_{11}$ receptor also sensitises prostate cancer and endothelial cells to mitoxantrone and cisplatin respectively, due to a decrease in Bcl-2 expression (Shabbir et al., 2008, Xiao et al., 2011). Thus, P2Y receptors could be used as a target in adjunct therapy to improve the efficacy of current chemotherapeutic agents.

1.5.7.2 Adenosine receptors

In addition to P2Y receptors, another subgroup of purinoceptor, namely adenosine receptors are also expressed in the endothelial cell environment (Feoktistov et al. 2002). Adenosine receptors are divided into four subtypes, namely A_1 and A_3 subtypes that couple to $G_{i/o}$ whereas A_{2A} and A_{2B} subtypes couple to G_s (Fredholm et al., 2011). Using RT-PCR to study the expression profile of adenosine receptors in endothelial cells, A_{2A} and A_{2B} receptor subtypes are shown to be the most abundantly expressed (Feoktistov et al., 2002, Iwamoto et al., 1994, Olanrewaju et al., 2000). Thus the following sections will focused on the discussion of the signalling pathways activated by these two receptor subtypes.

1.5.7.2.1 The role of adenosine receptors in atherosclerosis

Since ATP can be readily degraded *in vivo* to activate adenosine receptors, it is worthwhile considering the role of these receptors in the development of both cardiovascular disease and cancer. Early studies performed *in vivo* established a vital role for adenosine in the cardiovascular system as a potent vasodilator with increased cardiac adenosine concentrations (from 43 to 3700pmol/min in guinea pig heart)

detected under hypoxia conditions (Berne, 1963, Schrader et al., 1977, Deussen et al., 1989). Adenosine is released through the myocardial cell membrane (as detected through adenosine-8-C^{14}) (Jacob and Berne, 1960) or generated extracellularly from ATP via ecto-nucleotidase activity. Various studies have shown that adenosine acts on A_{2A} and A_{2B} receptors to play an important role in vasodilatation (Frobert et al., 2006, Hodgson et al., 2007, Talukder et al., 2003). For example, A_{2A} receptor activation induces coronary relaxation via p38 MAPK activation (Teng et al., 2005) and nitric oxide release (Teng et al., 2008), but the exact A_{2B} receptor mechanism underpinning this effect remains uncertain.

The use of knockout models for adenosine receptor subtypes confirms their role in atherosclerosis. Compared to wild type, $A_{2A}^{-/-}$ mice display higher levels of aggressiveness, a slower response to heat stimuli and increased platelet aggregation, blood pressure and heart rate (Ledent et al., 1997). Isolated aortic tissues from the A_{2A} knockout mice also show a reduction in relaxation in response to acetylcholine (Ponnoth et al., 2009). These results correlate with the effect of an A_{2A} agonist in reducing the secretion of thrombospondin 1 in endothelial cells to increase angiogenesis and wound healing (Desai et al., 2005, Montesinos et al., 2004). On the other hand, a knockout mouse model for A_{2B} shows increased aortic lesion formation and leukocyte adhesion, with characteristics of low grade inflammation exemplified by higher levels of TNF-α, IL-6, ICAM-1, chemokine receptor type 4 (CXCR4), E- and P-selectin (Yang et al., 2008, Yang et al., 2006). Collectively, both A_{2A} and A_{2B} receptor subtypes play an important role in vascular disease and inflammation.

1.5.7.2.2 The role of adenosine receptors in cancer

All subtypes of adenosine receptor are found in cancers cells, as demonstrated using RT-PCR (Panjehpour and Karami-Tehrani, 2007). Amongst the adenosine receptor subtypes, only the role of A_{2A} and A_3 receptors in cancer are well studied (Gessi et al., 2011). The use of A_{2A} antagonism as a cancer immunotherapy has been proposed based on the finding that adenosine inhibits the anti-tumour effect of T cells (Ohta

and Sitkovsky, 2009, Sitkovsky et al., 2008). Deletion of A_{2A} completely blocks melanoma tumour growth to promote host mice survival, possibly via upregulation of interferon-γ production by anti-tumour T cells (Ohta et al., 2006). On the other hand, increased protein expression and mRNA levels of A_3 receptor in cancer tissues has been found to correlate with cancer severity in colon, breast, thyroid, melanoma and pancreatic cancer (Fishman P, 2009). However, application of an A_3 agonist produces both induction and inhibition in cancer cell proliferation, depending on the cell type. For instance, growth inhibition has been reported in breast cancer and melanoma (Merighi et al., 2005, Panjehpour and Karami-Tehrani, 2007), but proliferation is observed in colon cancer (Gessi et al., 2007). Thus, future studies are still required to clarify the role of adenosine receptors in normal and cancer cells for the application of receptor modulators to be used clinically.

1.6 Aims and hypothesis of study

As discussed in section 1.5, various GPCRs have a regulatory role in reducing TNF-α mediated pro-inflammatory JNK signalling. This study attempts to elucidate the inhibitory potential of the ubiquitously expressed purinoceptors in both the cardiovascular endothelial cells (chapter 3) and breast cancer cells (chapter 4). It is hypothesised that activation of purinergic receptors could reduce cytokine and UVC mediated JNK activity. The downstream G-protein signalling components of purinergic receptors could mediate the inhibitory effects observed, to reduce inflammatory conditions.

This study aims to

(A) characterise the purinoceptor mediated inhibition of cytokine induced JNK signalling in endothelial cells; and examine both the cytokine and UVC mediated activation of JNK in cancer cells and the effect of purinoceptors have on this

(B) identify the signalling components involved in the inhibitory effect mediated by purinoceptors

(C) determine whether purinergic receptor mediated inhibition of JNK signalling could modify cellular outcomes relevant to disease pathology.

2.0 Materials and Methods

2.1 Materials

2.1.1 General reagents

All materials used in this study were of the highest commercial quality available and supplied from Sigma-Aldrich Co Ltd. (Poole, Dorset, UK) unless mentioned otherwise.

BD Biosciences (Oxfordshire, UK): PE Annexin V apoptosis detection kit I (BD Pharmingen)

Biorad Laboratories (Hertfordshire, UK): Pre-stained molecular weight markers for SDS-PAGE

GE Healthcare UK Ltd (Buckinghamshire, UK): Cellophane sheets, ECL detection reagents, glutathione sepharose 4B, nitrocellulose membrane (Hybond ECL)

Insight Biotechnology Ltd (Wembley, UK): Recombinant human TNF-α, recombinant human IL-1β

Roche Diagnostics Ltd (West Sussex , UK): Bovine serum albumin fraction V, 1.4-dithiothreitol (DTT)

2.1.2 Reagents for cell culture

Corning BV (Netherlands): All cell culture plasticware

Gibco BRL (Paisley, UK): Trypsin, antibiotics (penicillin streptomycin), foetal calf serum (FCS), L-glutamine, Dulbecco's modified eagles medium (DMEM)

Lonza Verviers SprL (Belgium): Endothelial basal medium (EBM-2) and single quote kit for EBM-2

2.1.3 Radiochemicals

PerkinElmer Life Science (Cambridge, UK): γ [^{32}P] ATP (3000 Ci/mmol)

2.1.4 Plasmids

Plasmid containing cDNA which encodes GST-tagged truncated c-jun N-terminus (GST-c-jun$_{5-89}$) was kindly provided by J.R. Woodgett from Ontario Cancer Instituite, Princess Margaret Hospital, Toronto, Canada.

2.1.5 Antibodies

Invitrogen Corporation (Camarillo, CA): Anti-phospho-p38 MAPK (rabbit polyclonal, 44-684G)

New England Biolab for Cell Signalling Technology, Inc (England, UK):

Anti-phospho-p65 (rabbit polyclonal, Ser-536, #3031), anti-phospho-SAPK/JNK (rabbit polyclonal, Thr-183/Tyr-185, #9251)

Santa Cruz Biotechnology Inc (CA, USA): Anti-p38 MAPK (rabbit polyclonal, N-20), anti-phospho-ERK1/2 (mouse monoclonal, E-4), anti-ERK1/2 (rabbit monoclonal, C-14), anti-IKB-α (rabbit polyclonal, C-21), anti-p65 (rabbit polyclonal, C-20)

2.2 Cell culture

All cell culture work was conducted under sterile conditions in a class II cell culture hood.

2.2.1 Human umbilical vein endothelial cells (HUVECs)

Primary HUVECs (approximately 500,000 cells per cryovial) were purchased from Cascade Biologics and maintained in endothelial basal medium (EBM-2) supplemented with single aliquots (10ml fetal bovine serum, 0.2ml hydrocortisone, 2ml recombinant human fibroblast growth factor (hFGF-B), 0.5ml recombinant human vascular endothelial growth factor (VEGF), 0.5ml recombinant human epidermal growth factor (rhEGF), 0.5ml recombinant long R insulin-like growth factor-1 (R3-IGF-1), 0.5ml gentamicin sulphate-amphotericin A (GA-1000), 0.5ml ascorbic acid and 0.5ml heparin). Medium was replaced every other day until the cells were utilised. Cells were subcultured after treating with trypsin solution.

2.2.2 Breast cancer cells MDA-MB-231

MDA-MB-231 cells were a kind gift from Dr Marie Boyd (Reader, University of Strathclyde, Glasgow). Cells were maintained in DMEM supplemented with penicillin (250 units/ml), streptomycin (25 mg/ml), L-glutamine (27 mg/ml) and FCS at 10%. Medium was replaced every other day until the cells were utilised. Cells were subcultured after treating with 0.2% EDTA/PBS solution (versene). When plated cells reached a confluency of approximately 80%, cells were rendered quiescent overnight with non-FCS DMEM media prior to experimental stimulations.

2.3 Methods

2.3.1 JNK solid phase kinase activity assay

Detection of JNK activation in solid phase assay was based on the affinity of JNK to bind and form a stable complex with the transcription factor, c-Jun. The truncated N-terminus glutathione-S-transferase (GST)-c-jun$_{5-89}$ was used as a substrate for JNK that was immobilised on glutathione sepharose beads (GSH) (Smith and Johnson, 1988). This substrate bound specifically to JNK protein in the cell lysates, (Minden et al., 1994b, Dai et al., 1995), thus the phosphorylated c-jun detected from the ATP-^{32}P reaction was a direct measure of JNK activity.

2.3.1.2 Production and purification of GST-c-jun$_{5-89}$

Recombinant GST-c-jun bacterial constructs were streaked on agar plates containing ampicilin and left to culture overnight at 37 °C. A single bacteria colony was picked and transferred to a 5 ml culture of 2XYT broth (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl and 100 μg/ml ampicillin). This was incubated at 37°C, shaking orbitally overnight. The bacteria amplification process was continued by transferring the 5ml bacteria broth into another 500ml 2XYT broth. After undergoing overnight orbital shaking at 37°C, sufficient bacteria growth was determined as 1ml of broth read A_{600} between 0.6 – 0.8. Then, GST-c-jun protein synthesis was induced by adding 100 μM isopropyl thio-β-D-galactopyranosidase (IPTG) with 30°C incubation for 4 hours. The bacteria were harvested in pellet form through centrifugation of broth at 10,000 g for 15 mins at 4°C, which was then stored overnight at -20°C.

In order to purify the GST-c-jun protein from the bacterial pellet, 12.5 ml of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM benzamadine, 1 mM β-mercaptoethanol, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 200 μM PMSF, pH 8) was used to resuspend the pellet. All subsequent steps were performed at 4°C to prevent protein degradation. Further lysing of the bacteria involved freeze-thawing and probe sonication steps. The lysis suspension was frozen in a dry ice-

methanol bath and left to thaw at room temperature. This was followed by probe sonication at 2 x 20 seconds for 50% intensity and 2 x 20 seconds at 80% intensity.

Sample aliquots were collected during the purification steps as summarised in figure 2.1(B) to confirm the purity of the GST-c-jun beads. Triton X-100 was then added into the suspension to form a final concentration of 1% w/v and the mixture was rotated on the wheel for 45 minutes at 4 °C. During this incubation step, 1ml glutathione sepharose beads (GSH) was conditioned by two washes with 10ml lysis buffer, with 10,000 g centrifugation for 2 minutes between the washes. The solubilised mixture was centrifuged at 10,000 g for 15 minutes and its supernatant added to the GSH beads, followed by 1 hour of wheel rotation at 4°C. GSH beads were washed twice with 10ml lysis buffer to remove any unbound GST-c-jun before storing at 4°C, in lysis buffer.

2.3.1.3 Qualitative and quantitative analysis of GST-c-jun bound to the carrier beads

The next phase of the process involved the elution of GST-c-jun protein from the GSH carrier beads for Bradford assay quantification. Sample aliquots were continually collected during the protein purification steps for electrophoresis analysis later. Beads (40 µl) were removed from the bead volume and centrifuged at 13,000 x g for 2 minutes in an Eppendorf tube. After the supernatant was aspirated, 200 µl of elution buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, 10 mM reduced glutathione, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, pH 8) was added. After the mixture was rotated on the wheel for 1 hour at 4°C, the beads were centrifuged at 13,000 g for 2 minutes and the supernatant was kept as E1. This elution buffer step was repeated again with the second supernatant kept as E2.

A Bradford assay was then performed to measure the amount of GST-c-jun bound to GSH as reflected in the protein amount in E1 and E2. Increasing BSA concentrations,

ranging from 5 – 20 µg/ml (containing appropriate volume of elution buffer), was measured on the spectrophotometer at 595 nm, to generate a standard curve, in which to measure/calculate the c-jun protein. E1 and E2 solutions (0.5 to 5 µl) were diluted with 800 µl distilled water and 200 µl Bradford reagent and mixed thoroughly. The resulting absorbance values were read at 595 nm and the protein concentration (GST-c-jun) calculated against the standard curve of BSA.

Qualitative analysis of the GST-c-jun purification process was performed by running the sample aliquots through SDS-PAGE as described in section 2.3.2.2. First, the samples collected were added with the indicated volume of 1 x or 2 x Laemmli sodium dodecyl sulphate sample buffer as indicated in Figure 2.1(A). Samples were loaded into a 10% gel and run at 130 V for approximately 90 minutes. Instead of transblotting the gel as described in the Western blotting section, the gel was stained with Coomassie blue solution (80% w/v methanol, 20% w/v acetic acid, 0.1% w/v Coomassie blue) for an hour. For clearer visualisation of proteins, non-specific background staining was removed by washing the gel with destain solution (80% w/v methanol, 20% glacial acetic acid) every 20 minutes for 2 hours. The gel was finally sandwiched between two cellophane sheets for drying down in a gel drier (Hoefer Scientific Instruments, USA).

A

Lane	Sample	Sample volume (µl)	Volume of sample buffer (SB) added (µl)	
			2 x SB	1 x SB
1	Molecular weight marker	-	-	-
2	Homogenate	2	-	38
3	Pellet (post detergent sample)	2	-	38
4	Supernatant (post detergent sample)	10	10	-
5	Post-binding supernatant	10	10	-
6	Wash 1	10	10	-
7	Wash 2	10	10	-
8	Elution 1	20	20	-
9	Elution 2	20	20	-
10	Beads	10	10	-

B

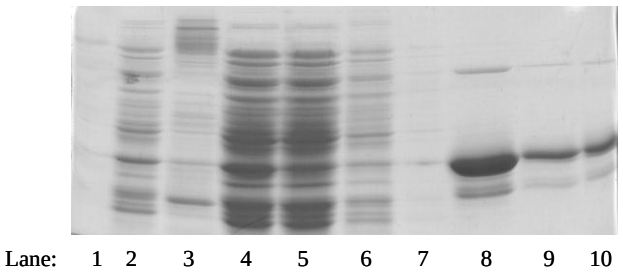


Figure 2.1: Gel electrophoresis of protein samples from various steps of the GST-c-jun purification process

Samples from various stages of the bead preparation process were collected as indicated in (A). They were loaded and run through gel electrophoresis as described in section 2.3.1.2 and shown in (B).

2.3.1.4 *In vitro* kinase assay

Confluent cells were stimulated for the respective experiments and the reaction was terminated by two washes of ice cold PBS. Then cells of each well were solubilised with 300 µl solubilisation buffer (20 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, 1% v/v Triton X-100, 0.5 mg/ml leupeptin, 0.5 mg/ml aprotinin, 0.5 mg/ml pepstatin, 0.1 mM sodium orthovanadate, pH 7.7), scraped and transferred into labelled Eppendorf tubes. The samples were vortexed briefly before leaving on ice for at least 30 minutes. At the same time, the mixture of tagged and untagged GST-c-jun GSH beads were prepared in a separate set of Eppendorf tubes, consisting of 13 µl of GSH-sepharose matrix (untagged carrier beads) and 20 µg of GST-c-jun tagged GSH beads. The bead mixture was then washed with 300 µl of solubilisation buffer, centrifuged and washed again. The earlier solubilised cell samples were centrifuged at 10,000 g for 5 minutes and the supernatants added to the beads for overnight mixing on the wheel at 4°C.

On the next day, the samples were centrifuged at 13,000 g for 5 minutes, followed by 300 µl solubilisation buffer and 300 µl kinase buffer (25 mM HEPES, 20 mM $MgCl_2$, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, pH 7.5) washes. The beads were resuspended in 25 µl of kinase buffer and 5 µl of ATP mixture containing γ-^{32}P (150 µM ATP and 0.5 µCi [γ-^{32}P] ATP) was added. The radioactive-ATP sample mixture was agitated at 30 °C for 30 minutes and the kinase reaction was terminated by the addition of 10 µl 4 x Laemmli sample buffer. Later, the samples were boiled for 5 minutes and centrifuged at 10,000 g for a minute. Samples were loaded onto a 10% SDS-PAGE gel and were run at 130 V for approximately 90 minutes.

The resulting gel was fixed in a solution containing 20% v/v methanol and 10% acetic acid for a minimum of 30 minutes, followed by sandwiching the gel in between two cellophane sheets in a drying frame for drying. The drying process was carried out at low heat (~70°C) for 90 minutes in a gel drier (Hoefer Scientific

Instruments, USA). Placing the gel in a metal cassette, the radio-labelled proteins were detected via exposing an autoradiography film for approximately 3 hours in -80°C. The film was developed with KODAK M-35M X-OMAT processor and the blots were semi-quantified through densitometry analysis as described in section 2.3.5.

2.3.2 Western blotting

Western blotting allowed the detection of proteins in a sample after its denaturation and separation according to molecular weight via the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system. The proteins were then transferred onto a membrane, followed by protein detection via the interaction of specific primary antibody, secondary enzyme-labelled antibody and chemiluminescence substrate.

2.3.2.1 Sample preparation for SDS-PAGE and immunoblotting

Confluent cells were stimulated for the respective experiments and the reaction was terminated by two washes with ice cold PBS. Then cells were lysed through the addition of hot (~85 °C) Laemmli SDS sample buffer (63 mM Tris/HCl, 2 mM $Na_2P_2O_7$, 5 mM EDTA, 50 mM DTT, 10% v/v glycerol, 2% w/v SDS and 0.007% bromophenol blue, pH 6.8). Cell scraping was performed on ice and cell lysates were drawn repeatedly through a syringe to shear chromosomal DNA prior to transfer to Eppendorf tubes. Samples were boiled at 95°C for 5 minutes to denature proteins before being stored at -20°C until required for analysis.

2.3.2.2 SDS-PAGE gel electrophoresis

The SDS-PAGE was prepared in a MiniPROTEAN^{TM} electrophoresis system. Initially, resolving gel containing 10% w/v acrylamide:bis-acrylamide (37.5:1) in 0.375 M Tris at pH 8.8, 0.1% w/v SDS, 0.5% ammonium persulphate (APS) and 0.05% v/v N, N, N', N'-tetramethylethylenediamine (TEMED) was prepared and

allowed to polymerise. A thin layer of 0.1% SDS was added above the resolving gel to prevent drying and disperse bubbles.

Once set, the SDS was washed away prior to addition of stacking gel with the appropriate comb slotted in to form 10 or 15 wells. The constituent of the stacking gel closely resembled the resolving gel, but with a different concentration of Tris at 125 mM, pH 6.8. When the stacking gel was completely polymerised, the comb was removed and its wells washed with distilled water. Gels were then transferred into a Western blot tank and running buffer (25 mM Tris, 192 mM glycine and 0.1% w/v SDS) was added. Loading of molecular weight markers and samples into the wells was performed using a Hamilton micro-syringe, followed by gel electrophoresis at 120 V for approximately 115 minutes.

Applying the transverse electrophoresis principle to transfer separated proteins onto a nitrocellulose membrane (Towbin et al., 1979), a sponge-filter paper-membrane-gel-filter paper-sponge sandwich was made in a cassette. The cassettes were placed in a Biorad mini trans-blot electrophoresis tank filled with an ice pack and transfer buffer (25 mM Tris, 192 mM glycine in 20% v/v methanol). After transblotting the gel at 280 mA for 2 hours, the membrane was blocked with 2% BSA in NaTT (20 mM Tris base, 150 mM NaCl and 0.2% Tween 20, pH 7.4) for 2 hours at room temperature. Next, the BSA was washed off before an overnight incubation of the appropriate primary antibody at an appropriate concentration in 0.2 % BSA/NaTT.

On the next day, membrane washing with NaTT was performed every 15 minutes for 90 minutes, followed by incubation of an appropriate horse-radish peroxidase conjugated secondary antibody in 15 ml of 0.2% BSA/NaTT for 90 minutes at room temperature, shaking. The membrane washing step was then repeated for 90 minutes. Finally, the membrane was treated with electrogenerated chemiluminescence (ECL) solutions for 2 minutes. In the darkroom, film was exposed to the membrane and

processed through an X-OMAT machine (Kodak M35-M X-OMAT). The processed membrane could then be stored in cold NaTT until stripping was performed as described below.

2.3.2.4 Stripping and reprobing of nitrocellulose membrane

Within a week of gel electrophoresis, stripping of antibody from the membrane could be carried out by incubating the membrane in stripping buffer (62 mM Tris HCl, 70 mM SDS, 0.1 M β-mercaptoethanol, pH 6.7) for 1 hour at 60 °C on a rotating shaker. The membrane was then washed every 10 minutes with NaTT for four times before overnight incubation with primary antibody in 2 % BSA/NaTT. Then the processing of membrane, including the 6 x NaTT washes, secondary antibody incubation, 6 x NaTT washes and enhanced chemiluminescence (ECL) processing, as described in section 2.3.2.2.

2.3.3 Scanning and densitometry

The blots were scanned with an Epson perfection GT-20000 scanner using Epson Scan Professional Mode software, while quantification of band intensity and normalisation of images against the background noise was performed using Scion Image (Scion Corp., Maryland, USA).

2.3.4 Flow cytometry analysis of apoptotic cells

Cells were analysed in accordance to the manufacturer's protocol for phycoerythrin (PE) Annexin V apoptosis detection kit I (BD Pharmingen). Cells were stained with PE annexin V and 7-amino-actinomycin (7-AAD) that binds to the phosphatidylserine at the exposed plasma membrane phospholipid and double-stranded nucleic acid of the porous cells respectively (Moser et al., 2001). Unstained and single stained cells treated with hydrogen peroxide were used to set the appropriate gates for the apoptotic and necrotic regions in the flow cytometer for 10,000 events.

After stimulation, cells were harvested with versene treatment and collected into 15 ml tubes. Cells were then centrifuged at 800 g for 5 minutes to form a cell pellet. The cell pellet was washed twice with PBS, and then resuspended into 100 µl of 1× annexin binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). PE annexin V and 7-AAD were each added into the samples at 5 µl, prior to 15 minutes incubation in the dark. Another 400 µl of binding buffer was added into the samples and the samples were processed through the FACScan flow cytometer using the FACS Diva software (FACScan, BD Biosciences). The data were analyzed using the FACS Diva (BD Biosciences) software.

2.3.5 Statistical analysis

All data were expressed in mean ± standard error mean (SEM), with graphs drawn in Prism 4. One-way ANOVA with Dunnett's post test was used for statistical analysis using densitometry data, when appropriate. Data with $p < 0.05$ was defined as being statistically significant. The EC_{50} and IC_{50} values were calculated using Biograph, with the $IC_{50} = Rmin + (Rmax – Rmin) / (1 + x / EC_{50})^{p}$.

3.0 The inhibitory effect of P2Y receptor in cytokine mediated JNK activity in endothelial cells

3.1 Introduction

Since the cloning and classification of purinergic receptors into seven ionotropic ligand gated ion channels ($P2X_{1-7}$) and eight metabotropic G protein coupled P2Y receptors, numerous studies have been carried out over the years to understand the role of ATP in different cell systems. In this chapter, the role of P2Y receptors, in the regulation of cytokine signalling, was investigated in endothelial cells. The rationale for this approach is related to the development of cardiovascular diseases, including atherosclerosis. In this condition, a chronic inflammation of the endothelium underpins the development of a lesion at the blood vessel inner wall which ultimately detaches from the wall to obscure the blood supply to the heart, leading to heart ischemia and infarct (Ross, 1999). By understanding how inflammatory signalling by cytokines in endothelial cells can be inhibited by the activation of certain GPCRs, this may pave the way for the development of new and improved therapies.

Endothelial cells that line the inner lumen of blood vessels are exposed to changes in plasma contents, which vary according to the degree of inflammation and the exposure of cells in different locations to shear stress. Thus the endothelial cell layer is most vulnerable to the action of pro-inflammatory cytokines, reactive oxygen species, oxidised lipoproteins and extracellular ATP that are in the plasma or secreted locally either by macrophages or endothelial cells themselves. Endothelial dysfunction as part of the response-to-injury hypothesis (French, 1966, Virchow, 1856), involves pro-inflammatory mediator induced activation of signalling pathways such as MAPK, NF-κB and JAK-STAT (Hansson and Hermansson, 2011, Libby et al., 2002, Patel et al., 2000).

Several studies have shown that activation of a member of the MAPK cascade, JNK, in endothelial cells contributes to both inflammation and apoptosis during the development of atherosclerosis. Staining of the aortic arch from $ApoE^{-/-}$ mice detected increases in JNK phosphorylation in endothelial cells at the atherosusceptible site (ie greater curvature of aortic arch) compared to the atheroprotected site (Hahn et al., 2009). This higher JNK activity in the atherosusceptible site correlated with increased staining of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) in $ApoE^{-/-}$ mice compared to wild type (Orr et al., 2005). In addition, pre-incubation of HUVECs with the JNK inhibitor CT536706 reversed TNF-α mediated expression of vascular cell adhesion molecule-1 (VCAM-1), E-selectin, interleukin-8, caspase 3, protein phosphatase A (PP2A), TLR4 and RIP1 as measured by microarray, quantitative RT-PCR and Western blotting (Chaudhury et al., 2010).

Based on the multi-component nature of the progression of atherosclerosis, it is useful to examine the possible signalling interactions between these components. As shown in an early study in a hybrid endothelial cell model, EAhy926, pre-incubation of ATP inhibited TNF-α mediated JNK signalling (Paul et al., 2000). Thus, in this chapter, it is hypothesised that a similar inhibitory phenomenon could be observed in human primary endothelial cells. The specificity of the inhibitory effects of ATP in regulating JNK signalling by other cytokines, such as IL-1β, was also studied in depth. Furthermore, the signalling molecules involved in the inhibitory actions of ATP were investigated using pharmacological inhibitors and siRNA.

3.2 Characterisation of ATP mediated MAPK signalling in HUVECs

Incubation of ATP at a micromolar concentration range has been shown to cause subsequent MAPK signalling as reported in various studies on endothelial cells (Montiel et al., 2006, Patel et al., 1996, Short et al., 2000, Xiao et al., 2011). The initial part of this study focused on the understanding of kinetics and concentrations of ATP required for activation of the MAPK signalling pathway, namely JNK, p38 MAPK and ERK. The activation of JNK was measured by both *in vitro* kinase assay and Western blotting using phospho-JNK antibodies; whereas p38 MAPK and ERK phosphorylation was examined using phospho-p38 or phospho-ERK antibodies.

3.2.1 JNK signalling

Incubation of HUVECs with ATP (100 µM) produced a transient JNK signal that peaked at 15 minutes (6.98 ± 0.52 fold stimulation compared to unstimulated control, $p < 0.001$). By 30 minutes, the stimulation fell nearly to baseline (1.54 ± 0.31 fold stimulation) as shown in Figure 3.1. Using the time point that demonstrated a maximal JNK signal in response to ATP (15 minutes), the concentration dependent activation of JNK was measured in Figure 3.2. Results from both *in vitro* kinase assay and phospho-JNK immunoblotting demonstrated a concentration dependent increase in JNK activation by ATP over the concentration range of 1 to 100 µM, with a maximum response of approximately 4 fold of stimulation at 100 µM (kinase activity: 4.19 ± 0.63 fold stimulation, $p < 0.001$).

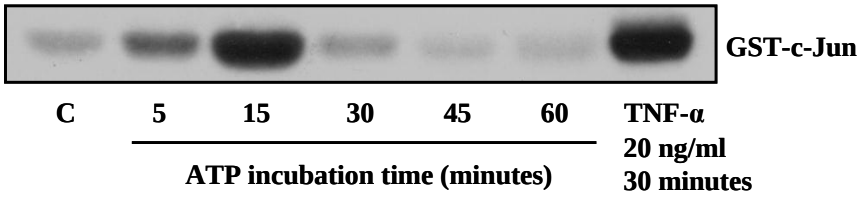
3.2.2 p38 MAPK signalling

A similar transient activation of p38 MAPK was observed following ATP stimulation of HUVECs (Figure 3.3). In contrast to JNK, p38 MAPK phosphorylation peaked earlier at 5 minutes (8.18 ± 1.94 fold stimulation compared to unstimulated control, $p < 0.01$) which then fell to less than half of the stimulation by 30 minutes (3.24 ± 0.51 fold stimulation). By 60 minutes, the p38 MAPK signal returned to lower than the baseline (0.52 ± 0.13 fold stimulation).

3.2.3 ERK signalling

In contrast to the transient JNK and p38 MAPK phosphorylation, ATP stimulation of HUVECs produced a more sustained activation pattern in ERK signalling as shown in Figure 3.4. ATP produced rapid ERK activation which was maximal by 5 minutes (5.09 ± 1.58 fold stimulation compared to unstimulated control, $p < 0.05$), which then declined gradually to approximately half by 45 minutes (2.71 ± 0.35 fold stimulation). By 60 minutes, ERK phosphorylation had returned to near baseline (1.23 ± 0.15 fold stimulation).

A



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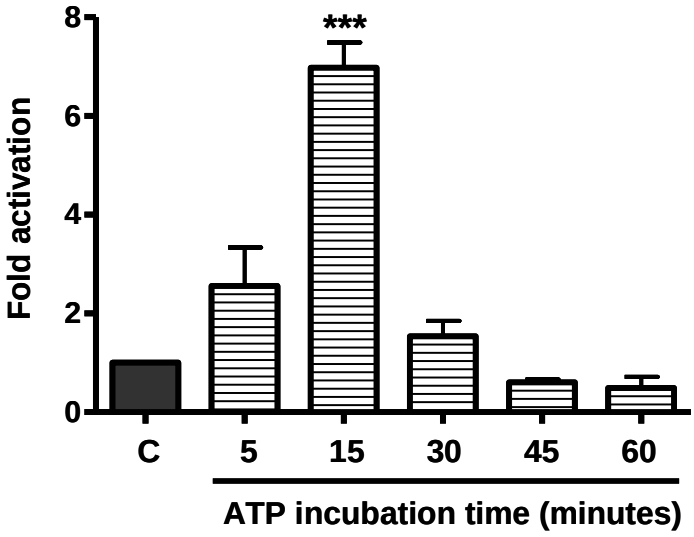
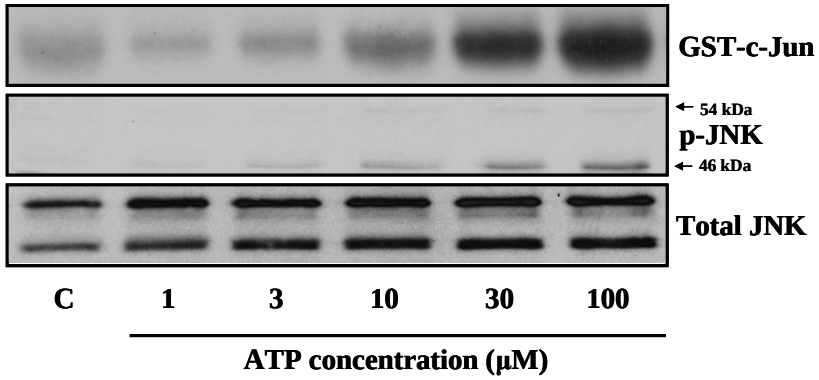


Figure 3.1: Time dependent JNK activation by ATP in HUVECs

Confluent cells were incubated with ATP (100 µM) for the times indicated above. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to unstimulated control.

A



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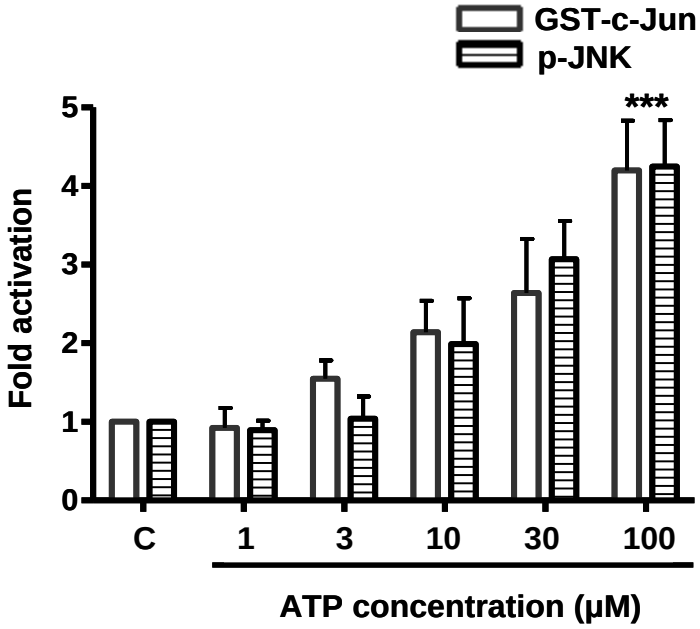
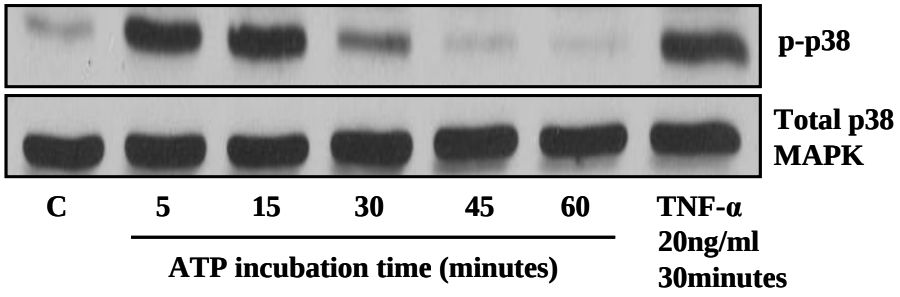


Figure 3.2: Concentration dependent JNK activation by ATP in HUVECs

Confluent cells were incubated with increasing ATP concentrations (1 - 100 μM) for 15 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to unstimulated control.

A



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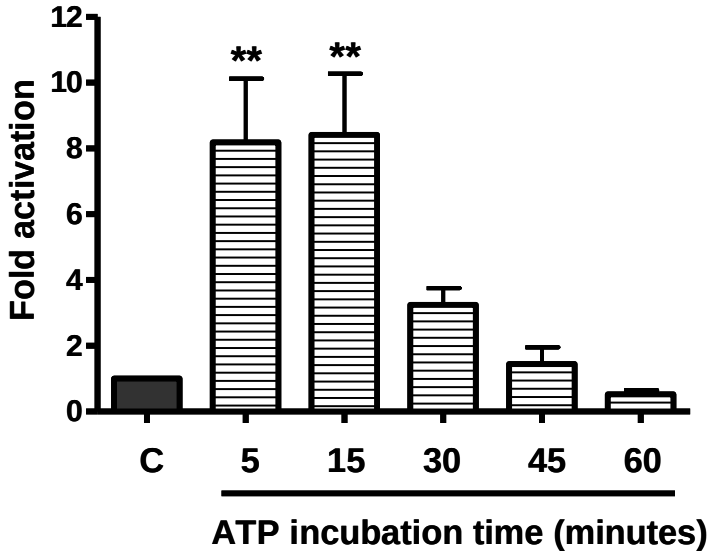

Figure 3.3: Time dependent p38 MAPK activation by ATP in HUVECs

Confluent cells were incubated with ATP (100 μM) for the times indicated above. Phosphorylation of p38 MAPK was assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to unstimulated control.

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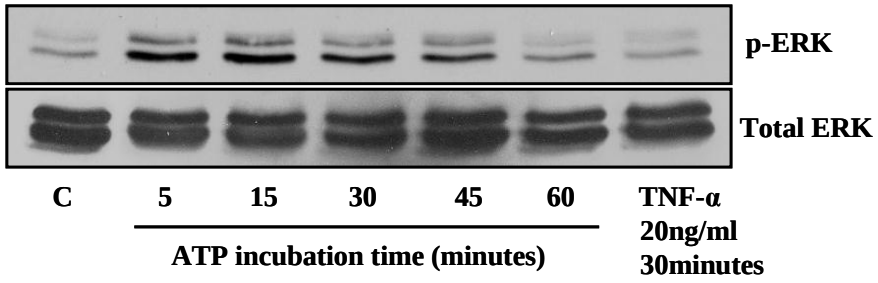

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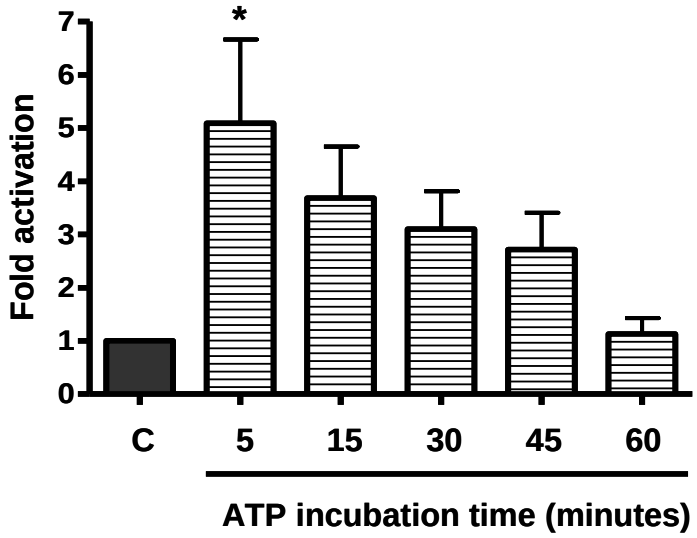

Figure 3.4: Time dependent ERK activation by ATP in HUVECs

Confluent cells were incubated with ATP (100 μM) for the times indicated above. Phosphorylation of ERK MAPK activity was assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. * $p < 0.05$ compared to unstimulated control.

3.3 Characterisation of TNF-α mediated MAPK signalling in HUVECs

Activation of the stress-activated protein kinase members, JNK and p38 MAPK, by cytokines such as TNF-α, has been well documented in endothelial cells (Karmann et al., 1996, Mong et al., 2008, Read et al., 1997, Wadgaonkar et al., 2004). Initially the kinetics and concentration dependency for TNF-α activation of JNK and p38 MAPK were established.

Stimulation of HUVECs with TNF-α (20 ng/ml) resulted in a rapid and significant increase in JNK activation, which peaked at 15 minutes (6.82 ± 0.80 fold stimulation compared to unstimulated control, $p < 0.001$). However the response was biphasic, decreasing between 30 and 60 minutes before increasing again at 90 minutes as shown in Figure 3.5.

In addition, activation of JNK by TNF-α was studied at the 30 minute time point as shown in Figure 3.6. In general, HUVECs were found to be highly sensitive to TNF-α, giving a substantial response as low as 1 ng/ml. Between 1 - 10 ng/ml, responses were comparable and only at 20 ng/ml was there a further significant increase in activity (5.63 ± 0.60 fold stimulation, $p < 0.01$). Thus TNF-α at 10 ng/ml for 30 minutes of incubation was chosen for the combined ATP stimulation studies, unless stated otherwise.

Similarly, TNF-α stimulated a strong increase in p38 MAPK phosphorylation in HUVECs as shown in Figure 3.7. A maximum response was observed at 15 minutes (6.40 ± 1.21 fold stimulation, $p < 0.01$), which reduced slightly by 60 minutes (3.92 ± 0.37 fold stimulation, $p > 0.05$).

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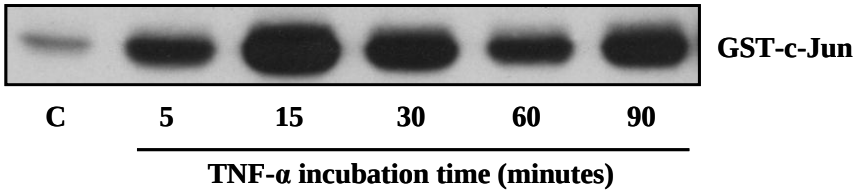

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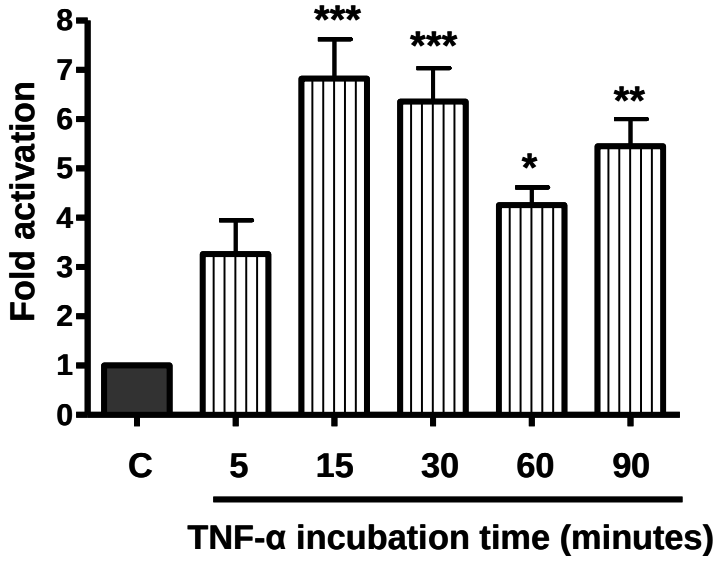

Figure 3.5: Time dependent JNK activation by TNF-α in HUVECs

Confluent cells were incubated with TNF-α (20 ng/ml) for the times indicated above. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to unstimulated control.

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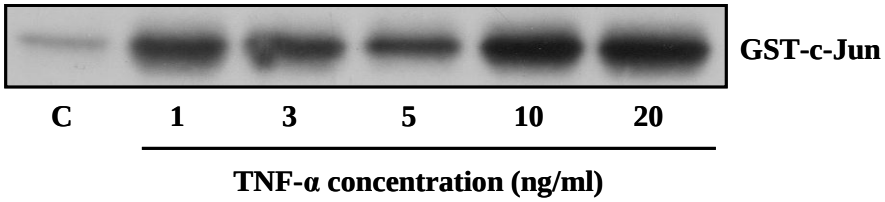

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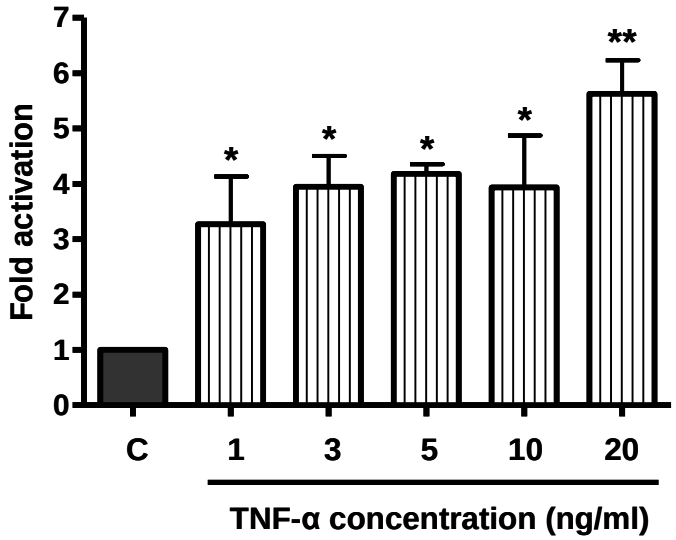

Figure 3.6: Concentration dependent JNK activation by TNF-α in HUVECs

Confluent cells were incubated with increasing TNF-α concentrations (1 – 20 ng/ml) for 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to unstimulated control.

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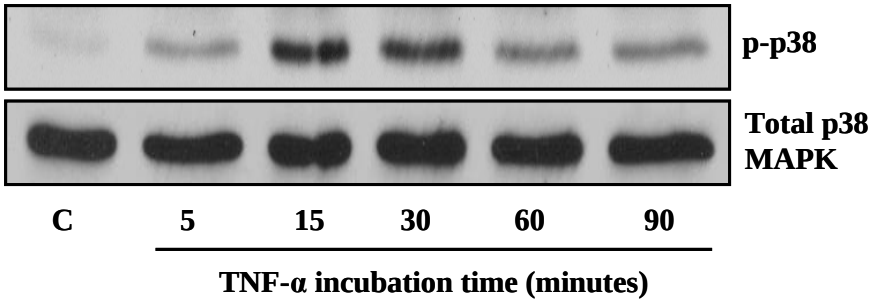

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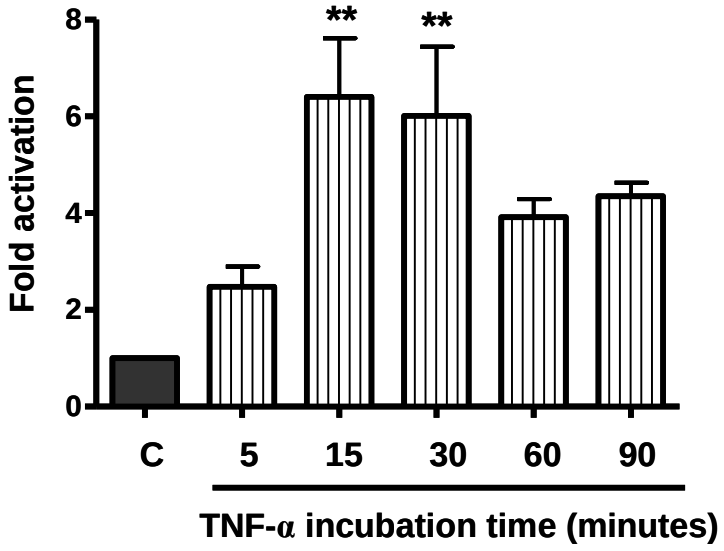


Figure 3.7: Time dependent p38 MAPK activation by TNF-α in HUVECs

Confluent cells were incubated with TNF-α (20 ng/ml) for the times indicated above. Phosphorylation of p38 MAPK was assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$ compared to unstimulated control.

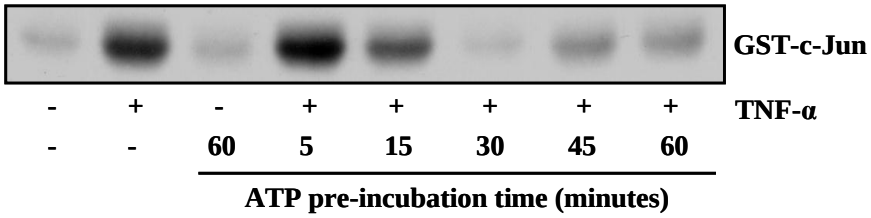
3.4 ATP mediated inhibition of TNF-α induced MAPK signalling in HUVECs

3.4.1 The kinetics and concentration dependency of ATP mediated inhibition of TNF-α stimulated JNK signalling

In order to characterise the effect of ATP in JNK signalling, time courses and concentration response experiments were conducted. Confluent HUVECs were exposed to ATP (100 μM) for 5 to 60 minutes, followed by TNF-α (20 ng/ml) incubation for another 30 minutes. ATP caused a time dependent decrease in TNF-α mediated JNK signalling as shown in Figure 3.8. Maximum inhibition of approximately 80% was observed following pre-incubation of ATP for 30 minutes (% TNF-α stimulation: ATP + TNF-α = 22.37 ± 2.13 %, $p < 0.05$), although some inhibition was observed as early as 15 minutes (Figure 3.8 panel A and C). Inhibition was maintained for up to 60 minutes of ATP pre-treatment, the maximum time point recorded. Furthermore, ATP alone was without effect on JNK signalling at any of the time points studied (Figure 3.8 panel B).

A concentration response relationship for ATP mediated inhibition of TNF-α induced JNK phosphorylation and activity was established for the 30 minute pre-incubation time point (Figure 3.9). ATP caused a concentration dependent inhibition of both JNK activity and phosphorylation which was maximal between 30 and 100 μM at approximately 40% of the initial TNF-α stimulation (% TNF-α stimulation at 100 μM ATP = 36.13 ± 4.97 % and 41.6 ± 5.17 %, $p < 0.05$). An IC_{50} value of approximately 8.6 μM was obtained over several kinase assay experiments.

A



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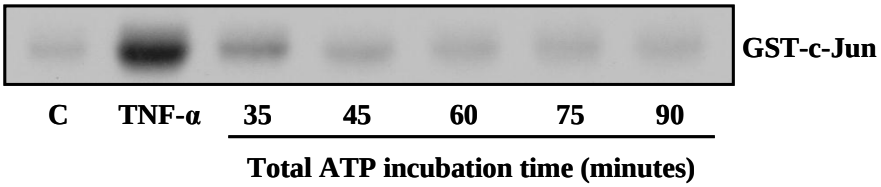

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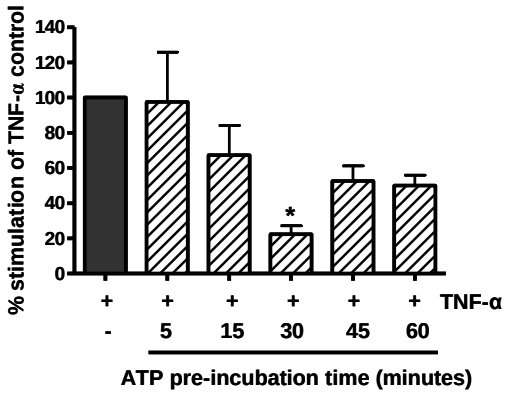

Figure 3.8: The kinetics dependency of the pre-incubation of ATP in TNF-α mediated JNK signalling in HUVECs

Confluent cells were incubated with ATP (100 µM) at the indicated durations (5 – 60 minutes), followed by TNF-α stimulation (20 ng/ml) for another 30 minutes as shown in (A). The JNK activity produced by ATP alone for the total duration of the combined stimulation was shown in (B). JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) and (B) showed representative blots and (C) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. * $p < 0.05$ compared to TNF-α only stimulation.

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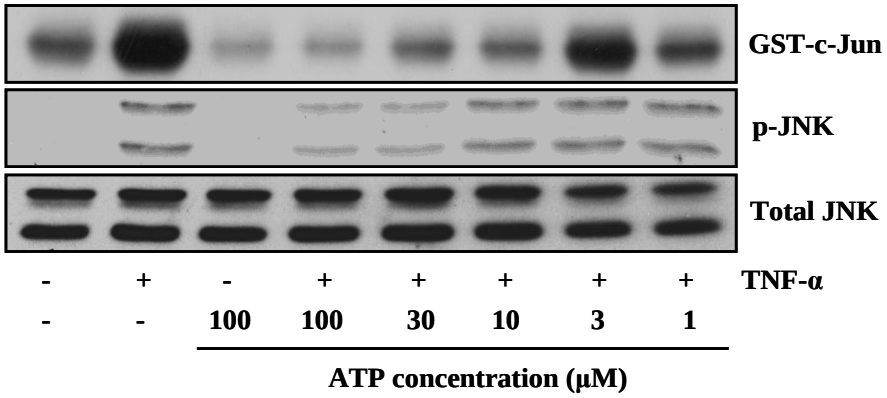

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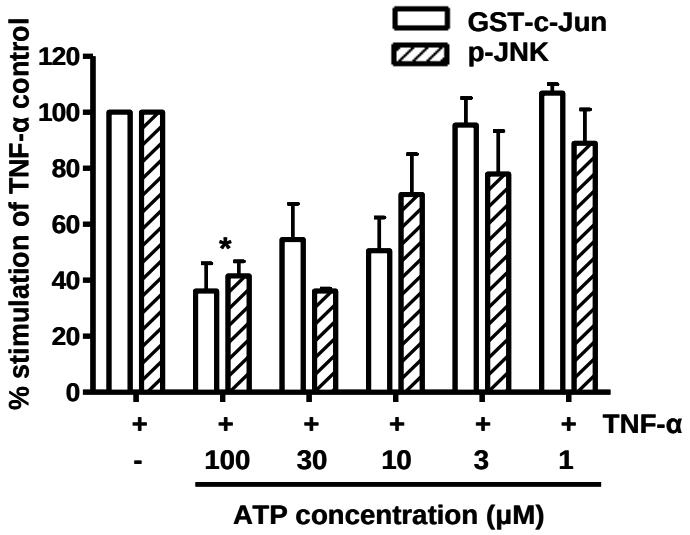


Figure 3.9: Concentration dependent ATP inhibition of TNF-α mediated JNK signalling in HUVECs

Confluent cells were incubated with increasing concentrations of ATP (1 – 100 μM) for 30 minutes, followed by TNF-α stimulation (10 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. * $p < 0.05$ compared to TNF-α only stimulation (GST-c-Jun and JNK phosphorylation).

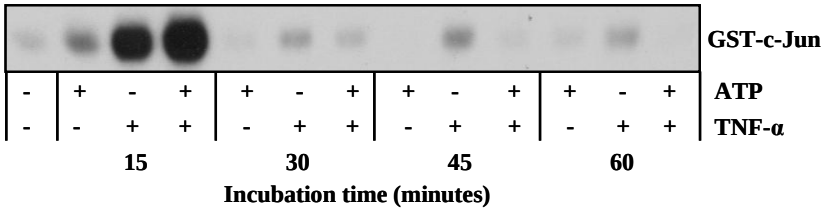
3.4.2 The effect of ATP and TNF-α co-stimulation on JNK signalling

To further explore the conditions whereby ATP could also inhibit TNF-α mediated JNK signalling, the effect of co-incubation with ATP (30 μM) and TNF-α (5 ng/ml) was examined over 2 hours. At 15 minutes, ATP alone produced a 3.54 ± 0.67 fold increase in JNK activity, whereas for TNF-α, the stimulation was higher at approximately 8 fold (8.15 ± 1.24 fold). Together there was a non-significant increase in JNK activity (9.31 ± 2.12 fold). In contrast, at later times, the declining TNF-α signal was reduced slightly but the difference was not statistically significant. This inhibition trend suggested that ATP was more effective when incubated with HUVECs prior to TNF-α stimulation.

3.4.3 The kinetics and concentration dependency of ATP mediated inhibition of TNF-α stimulated p38 MAPK signalling

As with section 3.4.1, similar experiments were performed to determine if pre-incubation of ATP could inhibit TNF-α mediated p38 MAPK. Addition of ATP (100 μM) for 5 to 60 minutes produced a minor and non-significant inhibition of TNF-α mediated p38 MAPK phosphorylation as shown in Figure 3.11 Panel A. At the pre-incubation time point of 30 minutes when maximum inhibition of JNK was observed, ATP did not alter the TNF-α mediated p38 MAPK phosphorylation (Figure 3.11 Panel C). These results suggested that the effect of ATP is largely selective for JNK inhibition.

A



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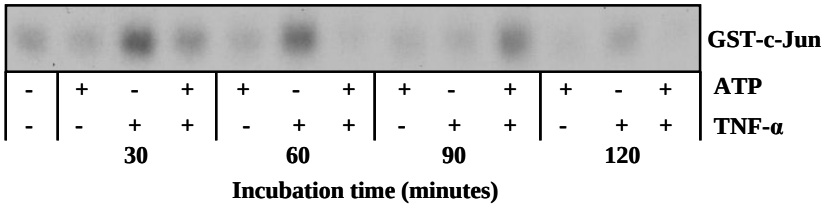

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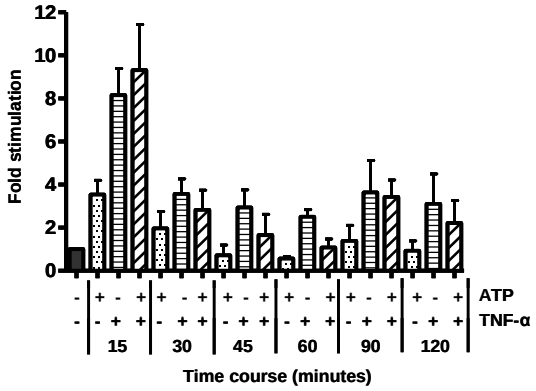

Figure 3.10: The effect of ATP and TNF-α co-stimulation on JNK signalling in HUVECs

Confluent cells were incubated with both ATP (30 µM) and TNF-α (5 ng/ml) for the indicated times (15 – 120 minutes). JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) and (B) showed representative blots and (C) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. No difference was found between TNF-α and ATP + TNF-α for the same time points, and the statistical differences between unstimulated control and ATP or TNF-α only stimulations were not indicated in the graph.

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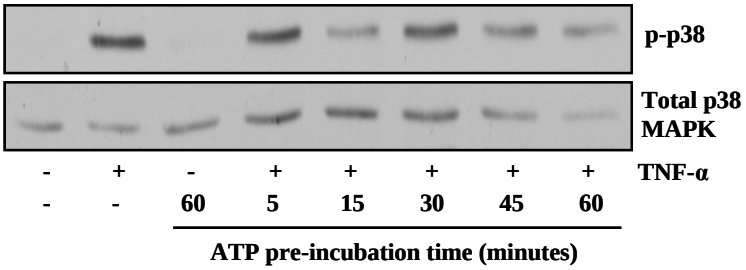

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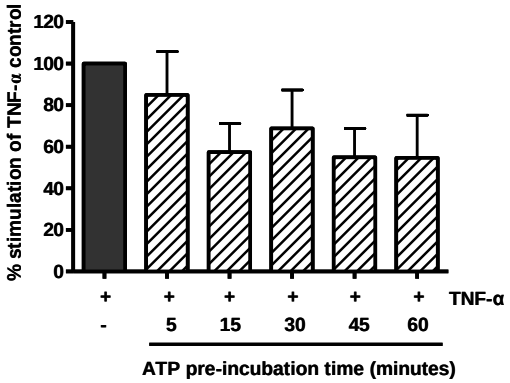

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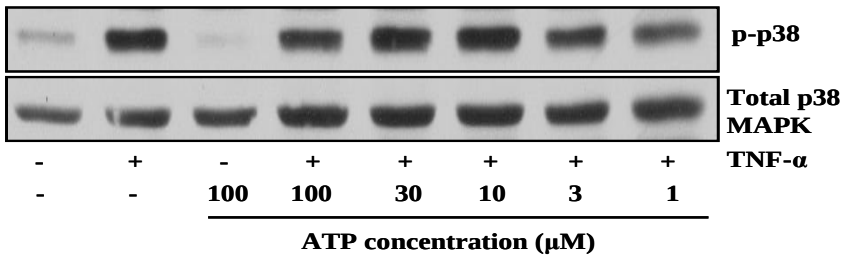

Figure 3.11: Kinetics and concentration study of the pre-incubation of ATP in TNF-α mediated p38 MAPK signalling in HUVECs

Confluent cells were incubated with ATP (100 μM) for the indicated times (5 – 60 minutes) in (A) or at increasing concentrations (0.1 – 100 μM) for 30 minutes in (B), followed by TNF-α stimulation (10 ng/ml) for another 30 minutes. Phosphorylation of p38 MAPK was assessed by Western blotting as described in section 2.6. (A) and (C) showed representative blots and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3.

3.5 ATP did not affect TNF-α mediated NF-κB signalling in HUVECs

Since JNK and NF-κB signalling can be regulated by common upstream intermediates, this part of the study sought to determine if ATP pre-incubation could also inhibit TNF-α mediated NF-κB signalling. Thus p65 NF-κB phosphorylation and IκB-α degradation were measured as the parameters for NF-κB activation. The effect of ATP and TNF-α alone or in combination was examined and results shown in Figure 3.12.

Stimulation of cells with ATP (100 μM) for up to 60 minutes did not result in any activation of NF-κB signalling as neither enhanced IκB-α degradation nor phosphorylation of p65 NF-κB was observed (Figure 3.12 Panel A). In contrast, incubation with TNF-α (20 ng/ml) activated NF-κB signalling with complete IκB-α degradation observed at 30 minutes and maximum phosphorylation of p65 NF-κB achieved at 15 minutes (approximately 5.5 fold) as shown in Panel B. The activation of NF-κB by TNF-α then declined gradually, both IκB-α and p65 NF-κB levels returned to baseline by 90 minutes. Thus a 15 minute TNF-α incubation was chosen to study the combined effect of ATP and TNF-α.

HUVECs were pre-incubated with ATP (100 μM) for up to 2 hours prior to stimulation with TNF-α for a further 15 minutes (Figure 3.12 Panel C). Alone, TNF-α as expected caused a substantial increase in p65 NF-κB phosphorylation, however this response was not affected by pre-incubation of ATP. These results strengthen the hypothesis that the inhibitory effect of ATP is pathway specific and does not involve NF-κB signalling.

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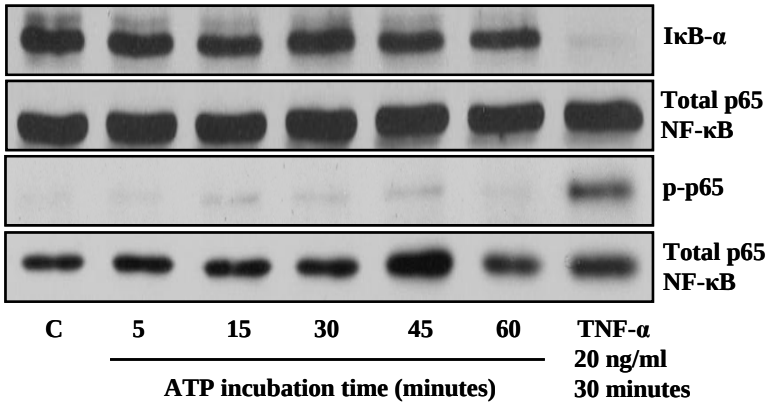

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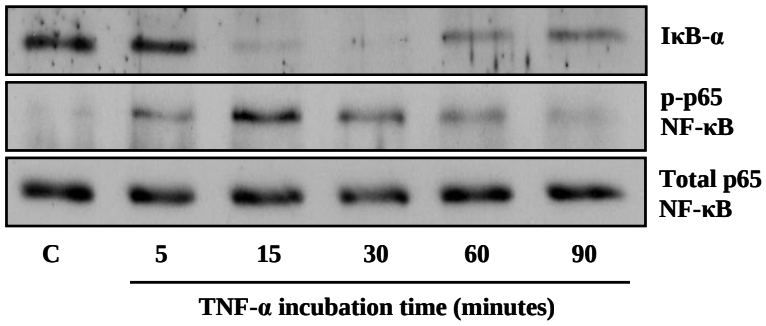

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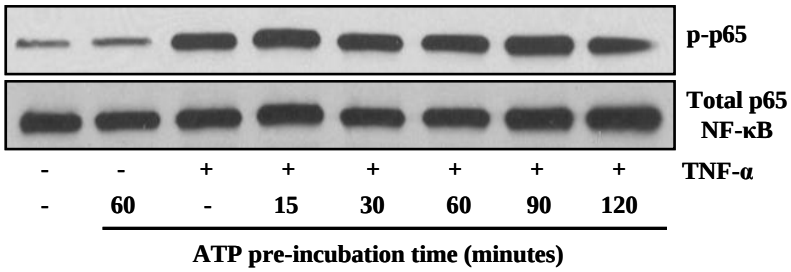

Figure 3.12: The effect of pre-incubation of ATP on TNF-α mediated p65 NF-κB activation in HUVECs

Confluent cells were incubated with ATP (100 µM) in (A) and TNF-α (20 ng/ml) in (B) for the times indicated above. ATP (100 µM) was pre-incubated for the indicated time points (15 – 120 minutes), followed by TNF-α stimulation (20 ng/ml) for another 30 minutes in (C). Degradation of IκB-α and phosphorylation of p65 NF-κB were assessed by Western blotting as described in section 2.6, n = 2.

3.6 ATP mediated inhibition of IL-1β induced MAPK signalling in HUVECs

The marked inhibitory effect of ATP on TNF-α mediated JNK signalling in HUVECs prompted further investigation of the specificity of ATP for other pro-inflammatory cytokines that are also involved in the pathogenesis of atherosclerosis and also utilises MAPK signalling pathways. Another cytokine, interleukin-1 beta (IL-1β), which utilises different adaptor proteins for pathway activation, was tested in place of TNF-α. Thus the MAPK activation by IL-1β alone was measured in order to choose the appropriate concentration and time point for the combined ATP and IL-1β experiments.

3.6.1 Characterisation of IL-1β mediated MAPK signalling

Similar to TNF-α stimulation, treatment of HUVECs with IL-1β (20 ng/ml) produced a transient and strong activation of JNK as shown in Figure 3.13. JNK signalling measured by both *in vitro* kinase assay and phospho-JNK immunoblotting reached peak activity at 15 minutes (kinase activity, fold stimulation: 7.01 ± 2.03, $p < 0.05$), that decreased slightly to approximately 5 fold at 30 minutes (5.54 ± 1.82 fold, $p > 0.05$). The JNK activity then fell rapidly to baseline by 90 minutes. Using the 30 minutes time point, a concentration curve for IL-1β stimulation (1 to 20 ng/ml) was generated as shown in Figure 3.14. A maximum increase in JNK kinase activity was observed between 5 and 10 ng/ml IL-1β with an EC_{50} value of 3.43 ± 1.74 ng/ml. Thus based on these results, IL-1β was used at 10 ng/ml over a 30 minute stimulation period for the following ATP pre-incubation studies.

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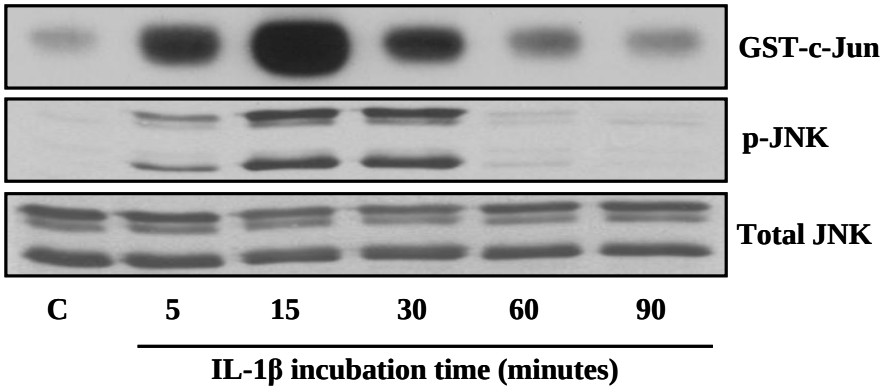

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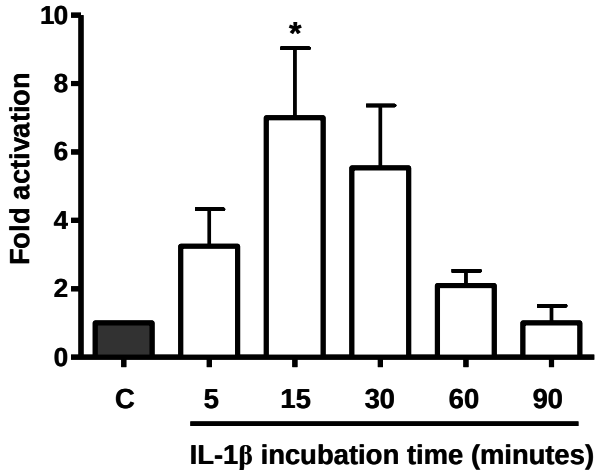

Figure 3.13: Time dependent JNK activation by IL-1β in HUVECs

Confluent cells were incubated with IL-1β (20 ng/ml) for the times indicated above. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data (GST-c-Jun phosphorylation) for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. * $p < 0.05$ compared to unstimulated control.

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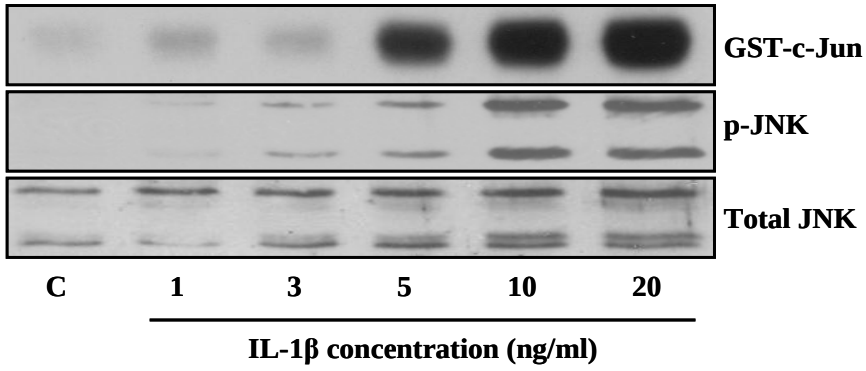

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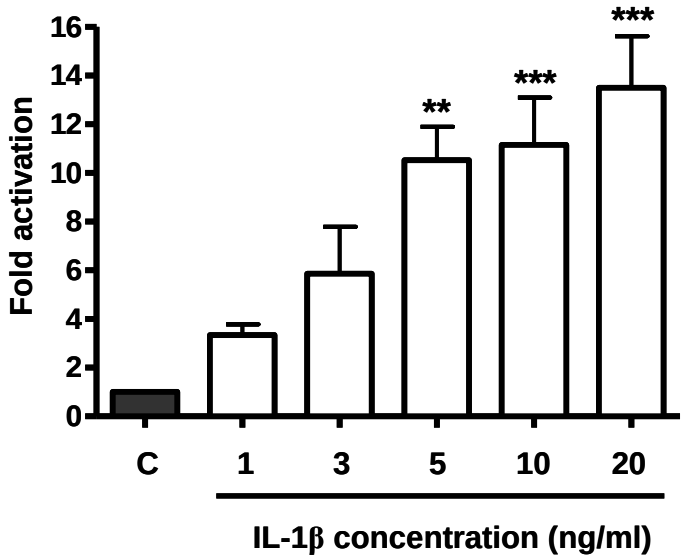

Figure 3.14: Concentration dependent JNK activation by IL-1β in HUVECs

Confluent cells were incubated with increasing concentrations of IL-1β (1 – 20 ng/ml) for 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data (GST-c-Jun phosphorylation) for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, ** $p < 0.01$ compared to unstimulated control.

3.6.2 Pre-incubation of ATP inhibited IL-1β mediated JNK signalling

In order to determine if ATP could inhibit IL-1β mediated signalling, the kinetics and concentration dependency of ATP in IL-1β induced JNK activity were examined. Alone, IL-1β stimulated a substantial increase in JNK activity in HUVECs as assessed by Western blotting of JNK phosphorylation. ATP caused a time dependent inhibition of IL-1β mediated JNK signalling, with maximum inhibition at 30 minutes (% IL-1β stimulation: ATP + IL-1β = 44.03 ± 7.70 %, $p < 0.01$) (Figure 3.15). The inhibitory effect of ATP was JNK specific, no effect upon IL-1β mediated p38 MAPK phosphorylation was observed at any time point tested (results not shown).

As shown in Figure 3.16, pre-incubation with ATP for 30 minutes produced a concentration dependent inhibition in IL-1β mediated JNK signalling as measured by both *in vitro* kinase assay and phospho-JNK immunoblotting. A maximum concentration of ATP (100 μM) produced approximately 70% inhibition of the IL-1β signal (% IL-1β stimulation: kinase activity: 31.11 ± 12.73 %, phospho-JNK: 32.53 ± 5.99 %, both at $p < 0.05$), with an IC_{50} of approximately 1 μM for the kinase assay results. Since ATP could inhibit JNK signalling mediated by both TNF-α and IL-1β, this suggests that the site of inhibition could be common for both cytokines.

3.6.3 The inhibitory effect of ATP at increasing concentrations of IL-1β

Next, the effect of a maximum concentration of ATP (100 μM) on increasing concentrations of IL-1β (1 – 20 ng/ml) was examined and the results shown in Figure 3.17. Despite the differences in the magnitude of JNK signalling by different IL-1β concentrations, ATP produced a largely consistent level of inhibition of over 60 %, although the IL-1β response was never totally abrogated. For example at 5 or 20 ng/ml of IL-1β, the inhibition was 57 % and 58 % (Fold stimulation: 5 ng/ml IL-1β = 9.44 ± 1.42, ATP + IL-1β = 4.02 ± 1.03, $p < 0.05$; 20 ng/ml IL-1β = 11.04 ± 0.24, ATP + IL-1β = 4.63 ± 0.91, $p < 0.01$). This indicates that ATP can only partially inhibit JNK signalling in response to IL-1β.

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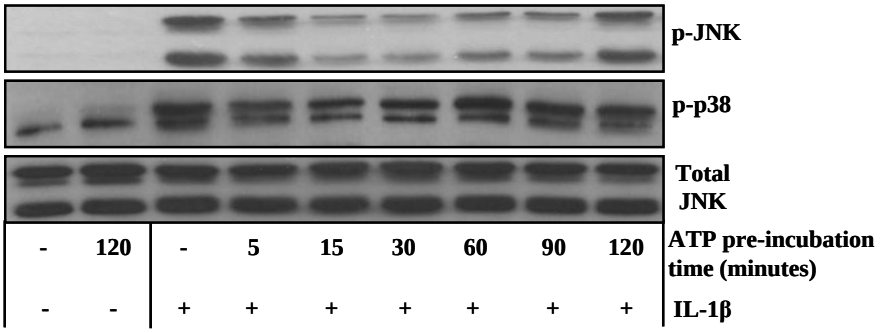

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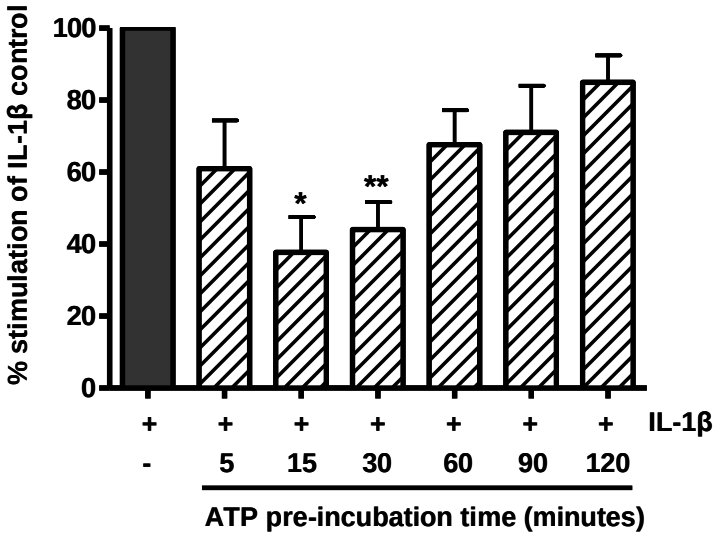

Figure 3.15: The kinetics of pre-incubation of ATP in IL-1β mediated MAPK signalling in HUVECs

Confluent cells were incubated with ATP (100 μM) at the indicated durations (5 – 120 minutes), followed by IL-1β stimulation (10 ng/ml) for another 30 minutes as shown in (A). Phosphorylation of JNK and p38 MAPK were assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data (JNK phosphorylation) for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to IL-1β only stimulation.

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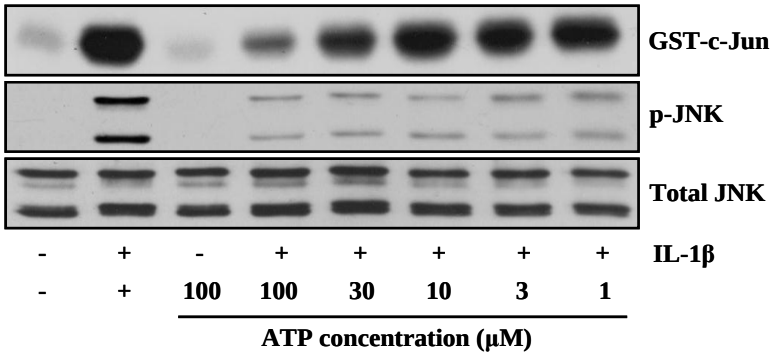

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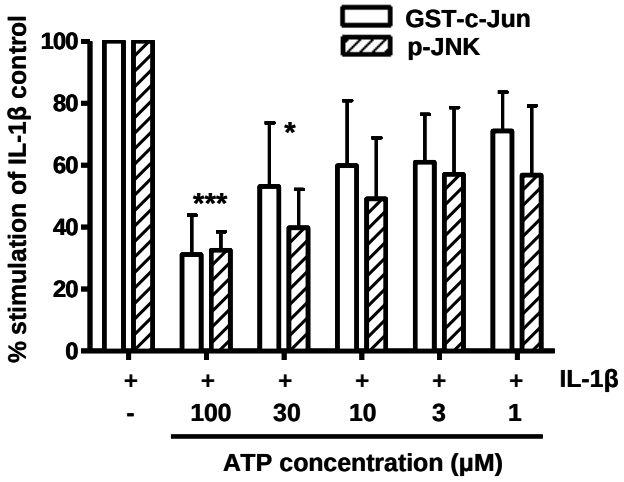

Figure 3.16: Concentration dependent ATP inhibition of IL-1β mediated JNK signalling in HUVECs

Confluent cells were incubated with increasing concentrations of ATP (1 – 100 μM) for 30 minutes, followed by IL-1β stimulation (10 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, * $p < 0.05$ compared to IL-1β only stimulation (GST-c-Jun and JNK phosphorylation).

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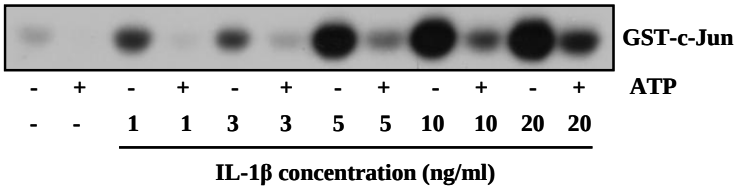

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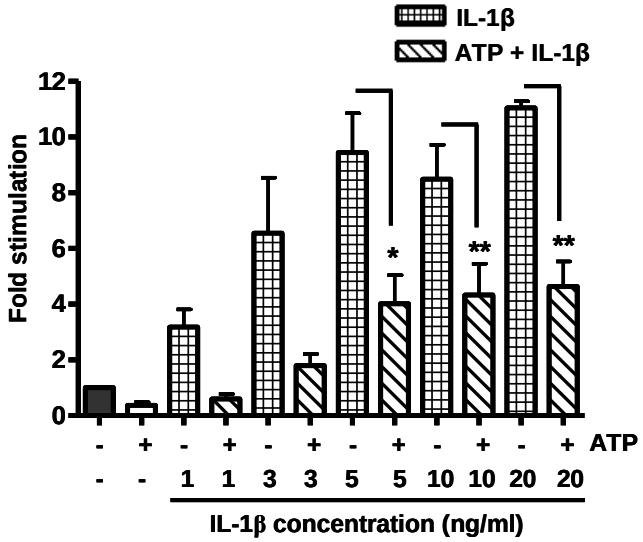

Figure 3.17: The inhibitory effect of ATP in JNK signalling mediated by increasing concentration of IL-1β in HUVECs

Confluent cells were incubated with ATP (100 μM) for 30 minutes, followed by increasing IL-1β stimulation (1 - 20 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to the respective IL-1β concentration control. The statistical differences between unstimulated control and IL-1β only or ATP + IL-1β stimulations were not indicated in the graph.

3.6.4 The effect of ATP and IL-1β co-stimulation on JNK signalling

In addition to pre-incubation studies, JNK activity following co-stimulation of ATP (30 μM) with IL-1β (5 ng/ml) was measured as outlined in Figure 3.18. Combined ATP and IL-1β stimulation produced higher JNK activity at 15 minutes, compared to IL-1β alone (Fold stimulation: ATP = 2.43 ± 0.49, IL-1β = 6.73 ± 1.78, ATP + IL-1β = 10.84 ± 1.73, $p < 0.05$). However at 30 minutes, combined ATP and IL-1β treatment reduced the JNK activity in contrast to IL-1β alone (Fold stimulation: ATP = 1.06 ± 0.32, IL-1β = 10.75 ± 0.61, ATP + IL-1β = 6.68 ± 0.86, $p < 0.01$). Longer incubation times up to 2 hours revealed a minor and insignificant increase in JNK activity for ATP and IL-1β in combination. These results demonstrate that inhibition of IL-1β mediated JNK signalling can occur irrespective of whether ATP was pre-incubated prior to IL-1β addition or post-30 minutes of simultaneous application.

3.7 ATP did not inhibit IL-1β mediated NF-κB signalling in HUVECs

Experiments in section 3.5 demonstrated that ATP only specifically inhibited JNK signalling, with no effect on NF-κB signalling. Thus a similar approach was applied to IL-1β stimulation and the results shown in Figure 3.19. As with TNF-α, IL-1β stimulated a marked increase in the degradation of IκB-α and enhanced the phosphorylation of p65 NF-κB. Pre-incubation of HUVECs with ATP for up to 120 minutes had no significant effect on either parameter stimulated in response to IL-1β at the 15 minute time point. Thus the inhibitory effect of ATP is specific for JNK signalling irrespective of the cytokine employed.

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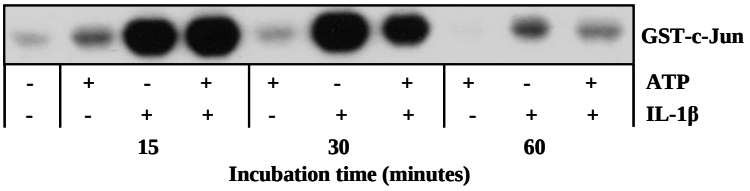

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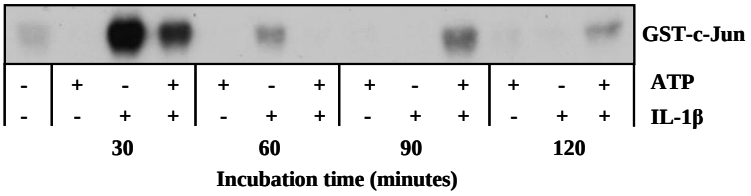

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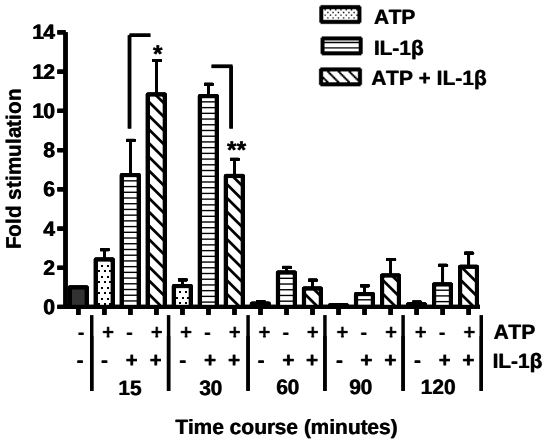

Figure 3.18: The effect of ATP and IL-1β co-stimulation on JNK signalling in HUVECs

Confluent cells were incubated with both ATP (30 μM) and IL-1β (5 ng/ml) for the indicated times (15 – 120 minutes). JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) and (B) showed a representative blot and (C) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to IL-1β only stimulation for the corresponding time points. The statistical differences between unstimulated control and ATP, IL-1β or ATP + IL-1β stimulations were not indicated in the graph.

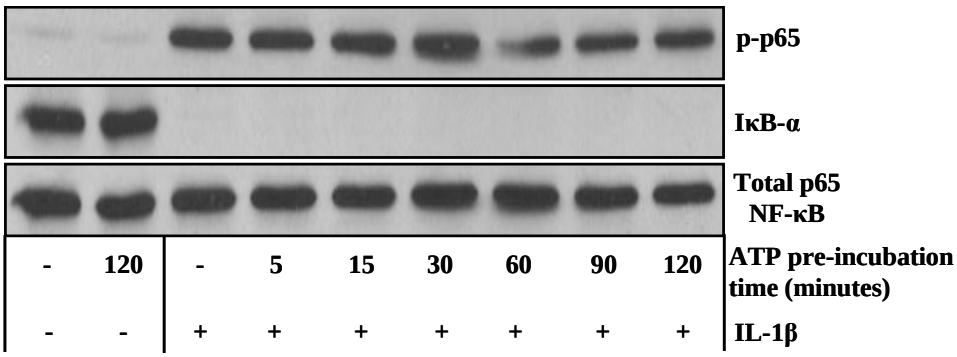

Figure 3.19: The effect of ATP pre-incubation on IL-1β mediated NF-κB activation in HUVECs

Confluent cells were incubated with ATP (100 μM) for the times indicated (15 – 120 minutes), followed by IL-1β stimulation (20 ng/ml) for a further 15 minutes. Phosphorylation of p65 NF-κB and degradation of IκB-α were assessed by Western blotting as described in section 2.6, n = 3.

3.8 The effect of ATP on JNK signalling in response to cellular stress

Since ATP inhibits the JNK signalling mediated by two different cytokines, the possibility that ATP could inhibit the JNK signalling in response to other agents known to induce JNK activation was then investigated. Previous studies have demonstrated that both anisomycin and hyperosmolarity induced by sorbitol are potent JNK activators (Bogoyevitch et al., 1995, Moriguchi et al., 1997, Raingeaud et al., 1995). Therefore HUVECs were pre-treated with ATP prior to the addition of anisomycin and sorbitol.

Preliminary studies demonstrated that incubation of HUVECs with sorbitol (0.5M) or anisomycin (0.5 μM) over a 90 minute period resulted in a substantial increase in JNK activity, equivalent to that observed in IL-1β (results not shown). Pre-incubation with ATP did not affect JNK phosphorylation or activity in response to either activator as shown in Figure 3.20. In contrast, ATP mediated a reproducible inhibition of IL-1β mediated JNK signalling. These results demonstrated that the mechanism of ATP mediated inhibition involves the upstream signalling components of cytokine stimulated pathways, and is not a general effect on components of the JNK pathway itself.

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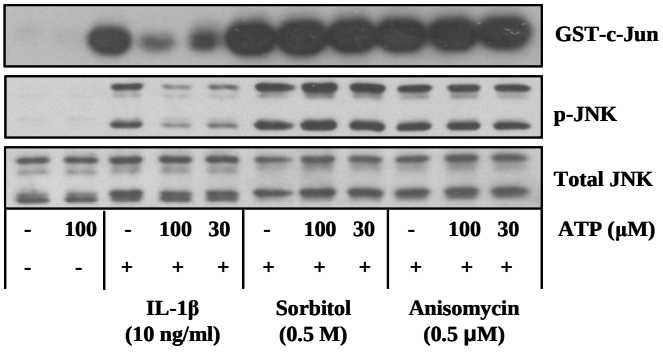

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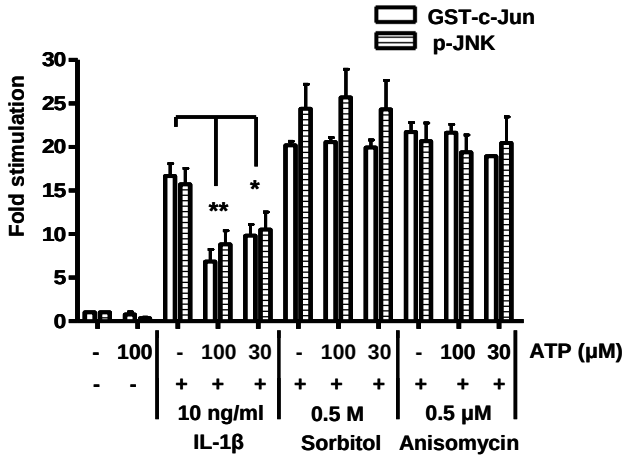

Figure 3.20: Specificity of ATP inhibition towards cytokine mediated JNK activation in HUVECs

Confluent cells were incubated with ATP (30 and 100 μM) for 30 minutes, followed by IL-1β (10 ng/ml), sorbitol (0.5 M) or anisomycin (0.5 μM) stimulation for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to IL-1β only stimulation. The statistical differences between unstimulated control and IL-1β, sorbitol, anisomycin or combined stimulations of ATP with these agents were not indicated in the graph.

3.9 Characterisation of the purinergic receptors involved in the inhibitory effects of ATP on JNK signalling on HUVECs

As outlined in the introduction, ATP mediates its cellular effects via a subgroup of GPCRs named P2Y purinergic receptors. A previous study in HUVECs utilising RT-PCR has shown that the most abundant P2Y is $P2Y_{11}$, followed by $P2Y_1$ and $P2Y_2$ receptors (Wang et al., 2002).Therefore receptor antagonists were employed to determine if either of these P2Y receptors mediated the inhibitory effect of ATP. The possible contribution of the degradatory products of ATP, such as ADP, AMP and adenosine, to its inhibitory effects was also evaluated by pre-incubation with an ecto-nucleotidase inhibitor.

3.9.1 The effect of $P2Y_{11}$ receptor antagonism in the ATP inhibition of IL-1β mediated JNK signalling

HUVECs were pre-incubated with the $P2Y_{11}$ receptor inhibitor, NF340 (Meis et al., 2010), prior to ATP pre-treatment and the resultant effect on IL-1β mediated JNK signalling was examined as shown in Figure 3.21. ATP at 10 μM mediated a 50% inhibition of IL-1β stimulated JNK activity, and this inhibition was partially reversed in the presence of NF340 at 10 μM (% IL-1β stimulation: ATP + IL-1β = 47.78 ± 8.00 %, NF340 10 μM + ATP + IL-1β = 88.71 ± 7.35 %, $p < 0.01$). The effect was concentration dependent as 3 and 1 μM of NF340 produced no significant reversal of inhibition. These data indicate that the inhibitory effect of ATP is likely to be mediated by $P2Y_{11}$ receptor in HUVECs.

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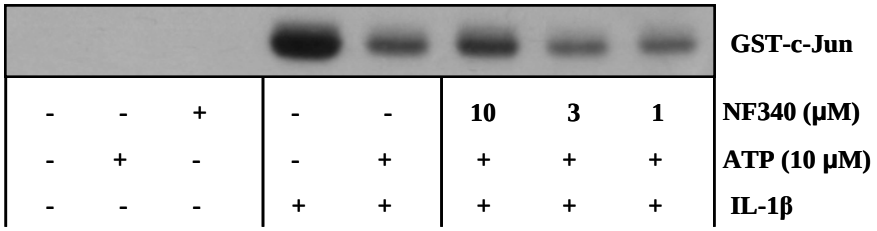

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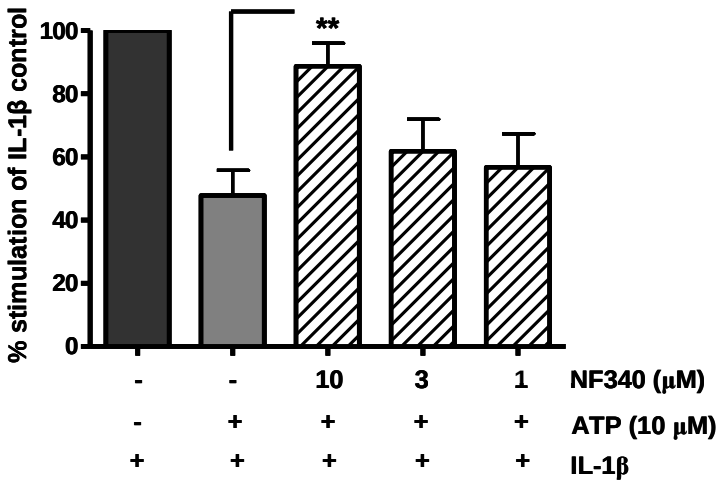

Figure 3.21: The role of $P2Y_{11}$ receptor in the inhibitory action of ATP for IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with increasing concentrations of NF340 (1 - 10μM) for 30 minutes, followed by ATP (10 μM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$ compared to the combined ATP and IL-1β stimulation. The statistical difference between IL-1β and ATP + IL-1β was not indicated in the graph.

3.9.2 The effect of $P2Y_1$ and combined $P2Y_1$ with $P2Y_{11}$ receptors antagonism in the ATP inhibition of IL-1β mediated JNK signalling

Since HUVECs also express high $P2Y_1$ receptor levels and $P2Y_1$ receptor is required for the internalisation of $P2Y_{11}$ receptor (Ecke et al., 2008), the role of $P2Y_1$ receptor alone and in combination with $P2Y_{11}$ receptor was tested using the $P2Y_1$ inhibitor, MRS2179 (Moro et al., 1998), as shown in Figure 3.24. Pre-incubation with ATP (30 μM) produced approximately 60% inhibition of IL-1β mediated JNK signalling, which was reversed slightly with prior treatment of the $P2Y_{11}$ antagonist, NF340 at 10 μM (% IL-1β stimulation: ATP + IL-1β = 40.19 ± 6.82 %, NF340 10 μM + ATP + IL-1β = 58.39 ± 8.87 %). Inhibition of $P2Y_1$ receptor alone by MRS2179 at 10 μM did not reverse the ATP mediated reduction of the IL-1β response (% IL-1β stimulation: ATP + IL-1β = 40.19 ± 6.82 %, 10 μM MRS2179 + ATP + IL-1β = 47.03 ± 8.13 %). Furthermore, combined $P2Y_1$ and $P2Y_{11}$ antagonism did not enhance the degree of reversal compared to $P2Y_{11}$ inhibition alone (% IL-1β stimulation: NF340 + ATP + IL-1β = 58.39 ± 8.87 %, NF340 + MRS2179 + ATP + IL-1β = 66.82 ± 8.15 %). These results demonstrated that the inhibitory effect of ATP is unlikely to be mediated by the $P2Y_1$ receptor.

3.9.3 Pre-incubation of UTP in IL-1β mediated JNK signalling: The role of the $P2Y_2$ receptor

A previous study in EAhy926 cells has demonstrated that $P2Y_2$ receptor mediated the inhibitory effect of ATP in TNF-α mediated JNK signalling (Paul et al., 2000). Since $P2Y_2$ receptor is also expressed in HUVECs and both ATP and UTP can equipotently activate the $P2Y_2$ receptor, the ability of UTP to inhibit IL-1β mediated JNK signalling was assessed (Figure 3.23). Pre-incubation of HUVECs with increasing concentrations of UTP (1 to 100 μM) affected neither JNK nor p38 MAPK signalling induced by IL-1β, suggesting that $P2Y_2$ receptor activation did not mediate the inhibitory effects of ATP in HUVECs.

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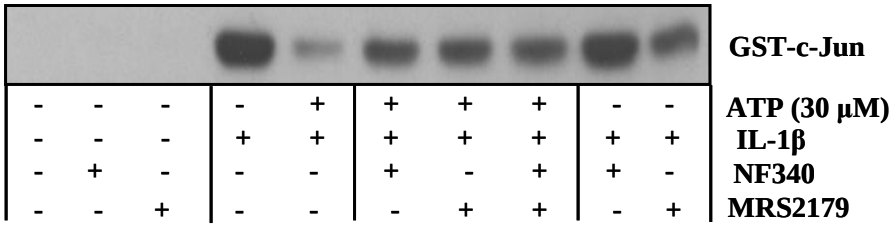

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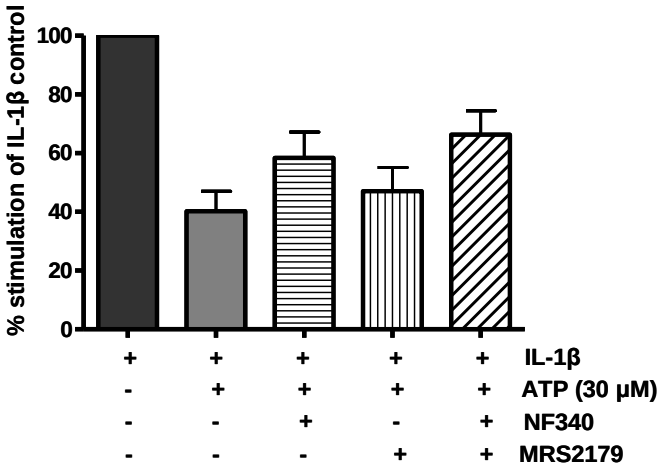

Figure 3.22: The role of $P2Y_{11}$ and $P2Y_1$ receptors in the inhibitory action of ATP for IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with NF340 (10µM) and/or MRS2179 (10 µM) for 30 minutes, followed by ATP (30 µM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no difference was found between ATP + IL-1β and other combined stimulations. The statistical difference between IL-1β only and combined treatments were not indicated in the graph.

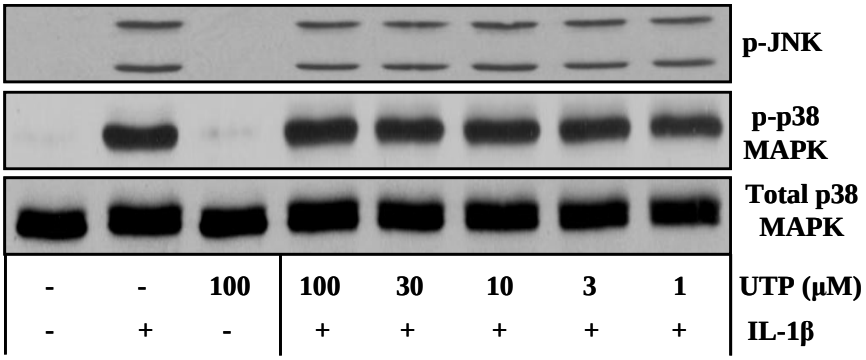

Figure 3.23: The role of $P2Y_2$ receptor in the inhibitory action of ATP on IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with increasing concentrations of UTP (1 – 100 μM) for 30 minutes, followed by IL-1β stimulation (10 ng/ml) for another 30 minutes. Phosphorylation of JNK and p38 MAPK were assessed by Western blotting as described in section 2.6, n = 2.

3.9.4 ATP degradation products did not contribute to the inhibitory effect of ATP in IL-1β mediated JNK signalling

The presence of ecto-nucleotidase on the cell surface allows rapid degradation of ATP into ADP and adenosine within minutes of ATP incubation (Wood et al., 2002). Therefore, there is a possibility that these degradatory products could also contribute to the inhibitory effects of ATP. As expected, pre-incubation of HUVECs with ATP (30 μM) inhibited IL-1β mediated JNK signalling by approximately 70 %, however pre-treatment with the ecto-nucleotidase inhibitor, ARL67156 (1 to 10 μM) (Chen and Lin, 1997) did not alter the inhibitory effect of ATP (% IL-1β stimulation: ATP + IL-1β = 35.31 ± 7.40 %, ARL67156 10 μM + ATP + IL-1β = 28.54 ± 8.14 %, $p > 0.05$) (Figure 3.24). In addition, ARL67156 alone affected neither basal kinase activity nor the response to IL-1β. This data suggests that ATP inhibition is unlikely to be dependent on its degradation products.

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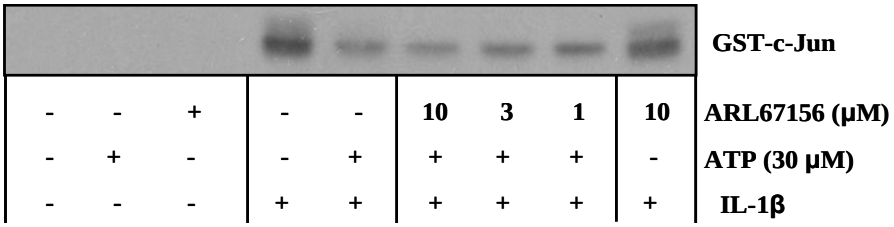

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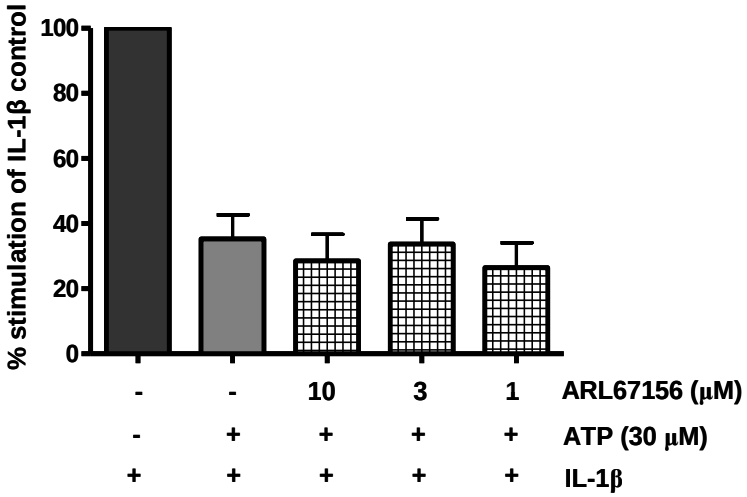

Figure 3.24: The role of ecto-nucleotidase in ATP mediated inhibition of IL-1β stimulated JNK activation in HUVECs

Confluent cells were incubated with ARL67156 (1 - 10μM) for 30 minutes, followed by ATP (30 μM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no difference was found between ATP + IL-1β and different concentrations of ARL67156 + ATP + IL-1β. The statistical difference between IL-1β only and combined treatment were not indicated in the graph.

3.10 Characterisation of signalling events involved in the inhibitory effect of ATP on JNK activity in HUVECs

As mentioned in the introduction (section 1.5.7.1), P2Y receptors are GPCRs which therefore couple to G proteins for their downstream signal transduction. Since the previous section has revealed that ATP mediated its inhibitory effects in JNK signalling via $P2Y_{11}$ receptor, the following sections then investigated the importance of different G proteins in mediating the inhibitory effect of ATP.

3.10.1 The role of $G_{q/11}$ in the inhibitory effect of ATP in cytokine mediated JNK signalling

In the following experiments, inhibition of $G_{q/11}$ activity was achieved by pre-incubation with the inhibitor YM254890. This compound has been shown to prevent GDP/GTP exchange on the $G\alpha_q$ subunit at nanomolar concentration range and has been widely used in various other studies (Takasaki et al., 2004). Thus the role of $G_{q/11}$ in the inhibitory effect of ATP in TNF-α and IL-1β mediated JNK signalling was tested as shown in Figure 3.25 and 3.26. In Figure 3.25, pre-incubation of ATP markedly reduced TNF-α mediated JNK phosphorylation and activity. Surprisingly, YM254890 at concentrations up to 100 nM had no significant effect on ATP (100 µM) mediated inhibition with the number of experiments performed (% maximal TNF-α stimulation: kinase activity: ATP + TNF-α = 26.64 ± 1.91 %, YM254890 + ATP + TNF-α = 53.58 ± 7.29 %).

In contrast, YM254890 mediated a reversal on the inhibitory effect of ATP (100 µM) on IL-1β induced JNK signalling, which was maximal between 30 and 100 nM of the compound (Figure 3.26). However at these concentrations the effect was still incomplete, reversal was approximately 50 % (% maximal IL-1β stimulation: kinase activity: ATP + IL-1β = 13.46 ± 3.15 %, YM254890 30 nM + ATP + IL-1β = 41.59 ± 11.80 %). Therefore, the inhibitory effect of ATP in cytokine mediated JNK signalling is not dependent on $G_{q/11}$ activity alone.

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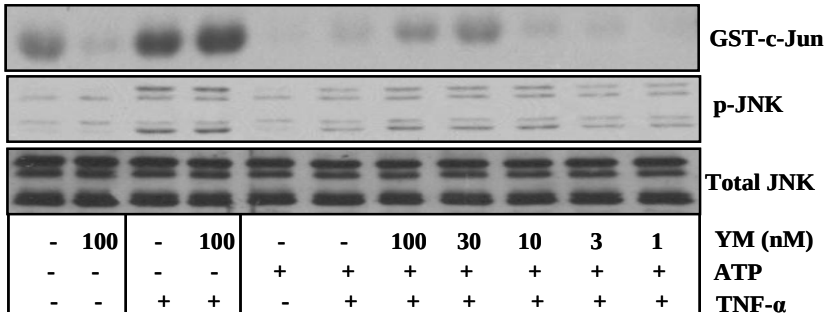

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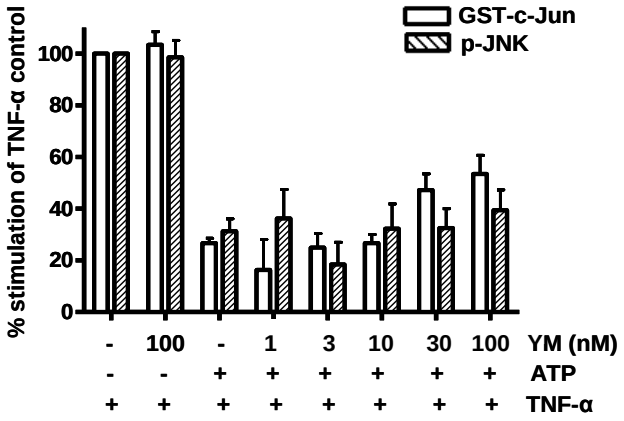

Figure 3.25: The role of $G_{q/11}$ in the inhibitory effect of ATP on TNF-α mediated JNK signalling in HUVECs

Confluent cells were incubated with increasing concentration of YM254890 (1 – 100 nM) for 15 minutes, followed by ATP (100 μM) for 30 minutes and TNF-α (20 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no difference was found between ATP + TNF-α and different concentrations of YM254890 + ATP + TNF-α. The statistical difference between TNF-α only and other treatments were not indicated in the graph.

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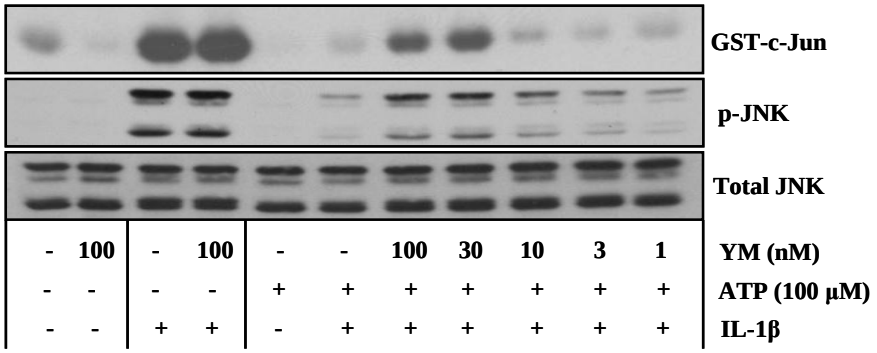

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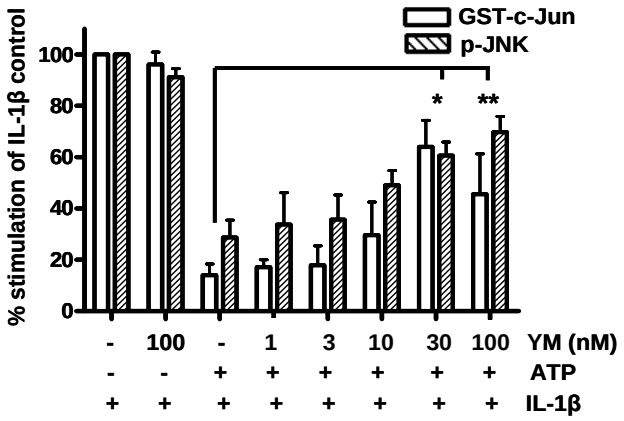

Figure 3.26: The role of $G_{q/11}$ in the inhibitory effect of ATP on IL-1β mediated JNK signalling in HUVECs

Confluent cells were incubated with increasing concentration of YM254890 (1 – 100 nM) for 15 minutes, followed by ATP (100 µM) for 30 minutes and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to combined ATP and IL-1β stimulation (GST-c-Jun and JNK phosphorylation). The statistical difference between IL-1β only and other treatments were not indicated in the graph.

3.10.2 The role of PKC in the inhibitory effect of ATP in IL-1β mediated JNK signalling

It is well established that PKC activation functions downstream of $G_{q/11}$ signalling. Several PKC isoforms are present in HUVECs as demonstrated by RT-PCR and immuno-staining, including α, β, ζ δ and ε (Dang et al., 2004, Ross and Joyner, 1997, Shizukuda et al., 1999). The role of PKC in the inhibitory effect of ATP was investigated by pre-incubation with broad spectrum PKC inhibitors, GF109203X and Go6976, as shown in Figure 3.27. As predicted, pre-incubation of ATP inhibited IL-1β mediated JNK signalling, however over the concentration range tested, neither PKC inhibitor altered the inhibitory effect of ATP (% maximal IL-1β stimulation: ATP + IL-1β = 30.13 ± 8.84 %, GF109203X 5 μM + ATP + IL-1β = 25.28 ±5.65 %; ATP + IL-1β = 41.33 ± 7.55 %, Go6976 10 μM + ATP + IL-1β = 39.91 ± 15.47 %; both $p > 0.05$). As these inhibitors have a higher selectivity towards the conventional PKCs (α and β), these results suggested that neither of these isoforms play a role in the regulatory effect of ATP.

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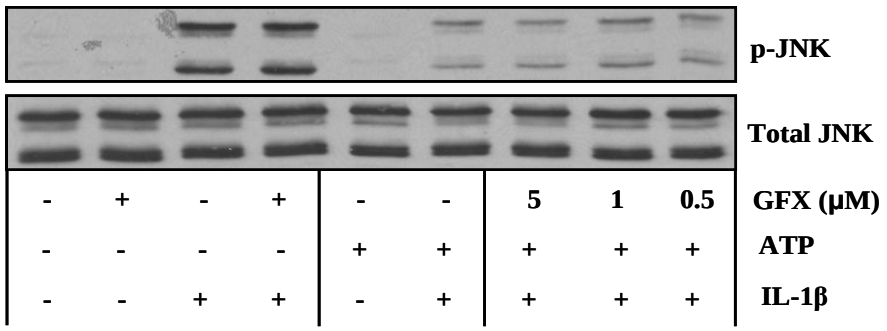

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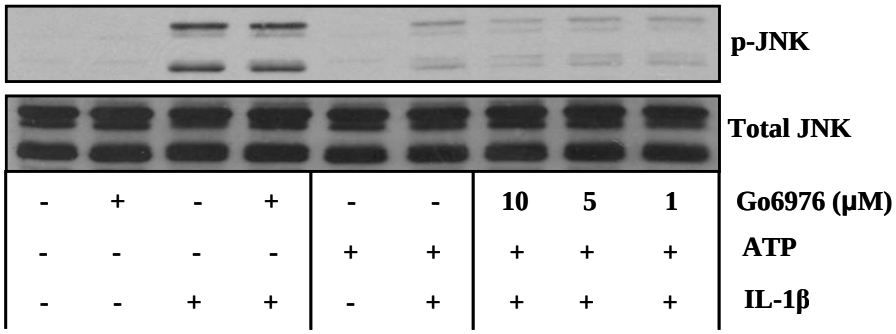

Figure 3.27: The general role of protein kinase C in the inhibitory action of ATP in IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with increasing concentration of GF109203X (0.5 – 5 μM) in (A) or Go6976 (1 – 10 μM) in (B) for 30 minutes, followed by ATP (100 μM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. Phosphorylation of JNK was assessed by Western blotting as described in section 2.6, n = 3.

3.10.3 The role of the G_s dependent pathway in the inhibitory effect of ATP in IL-1β mediated JNK signalling

As shown previously, inhibition of $G_{q/11}$ was found to be insufficient to reverse the inhibitory effect of ATP in IL-1β mediated JNK signalling, it is possible that other G protein dependent mechanisms could play a role. Previous studies have demonstrated that $P2Y_{11}$ receptor couples to both $G_{q/11}$ and G_s proteins (Communi et al., 1997). The roles of the G_s dependent intermediates, namely PKA and EPAC, were assessed. Adenosine receptor stimulation is known to strongly activate G_s via A_{2A} and/or A_{2B} receptors, which are expressed in HUVECs (Fang and Olah, 2007, Feoktistov et al., 2002, Montesinos et al., 1997). Thus, the effect of pre-incubation of adenosine was assessed to indirectly verify the ability of G_s to inhibit IL-1β mediated JNK signalling.

3.10.3.1 Characterisation of adenosine mediated inhibition of IL-1β induced JNK signalling

Preliminary studies demonstrated that incubation of HUVECs with adenosine (100 µM) alone over an hour did not produce any increase in JNK signalling (data not shown). The effect of pre-incubation with adenosine on IL-1β mediated JNK activity was studied as shown in Figure 3.28. Adenosine produced a concentration dependent inhibition of IL-1β mediated JNK signalling, measured by both *in vitro* kinase assay and Western blotting for JNK activity (% IL-1β stimulation: kinase activity: 32.13 ± 12.21 % and 35.42 ± 6.54 % at 100 and 30 µM adenosine, $p < 0.01$ for both). The calculated IC_{50} value for adenosine through kinase activity results was 5.55 ± 1.28 µM. These results demonstrated that G_s-coupled receptor activation could inhibit IL-1β mediated JNK signalling.

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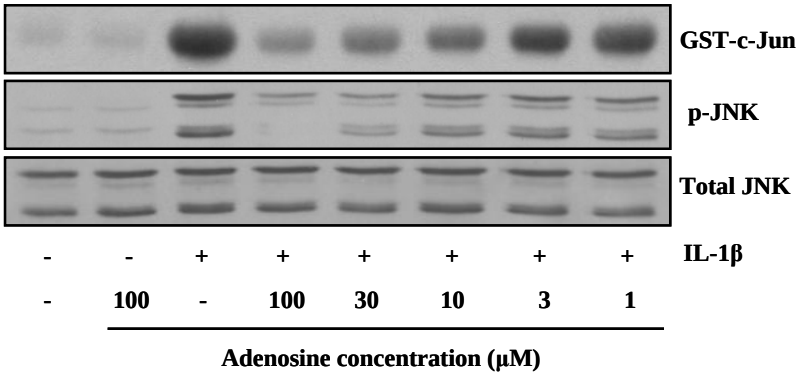

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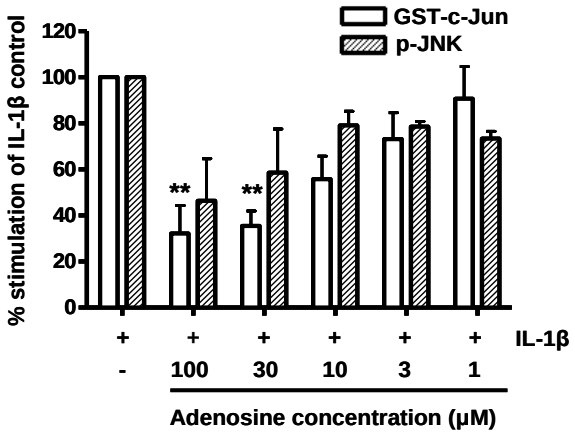

Figure 3.28: Concentration dependent inhibitory effect of adenosine in IL-1β mediated JNK signalling in HUVECs

Confluent cells were incubated with increasing concentrations of adenosine (1 – 100 μM) for 30 minutes, followed by IL-1β stimulation (10 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$ compared to IL-1β only stimulation (GST-c-Jun phosphorylation).

3.10.3.2 Characterisation of adenosine receptor(s) involved in the inhibitory effect of adenosine on IL-1β mediated JNK signalling

Subsequently, A_{2A} antagonist, ZM241385, was applied to study the role of A_{2A} receptor in the inhibitory effect of adenosine as shown in Figure 3.29. As observed in the previous subsection, pre-incubation of HUVECs with adenosine (10 μM) inhibited IL-1β mediated JNK activity (% IL-1β stimulation: adenosine + IL-1β = 58.79 ± 12.06 %), but this inhibitory effect was abolished in the presence of ZM241385 at 10 μM (% IL-1β stimulation: ZM241385 + adenosine + IL-1β = 105.60 ± 3.22 %, $p < 0.01$). This result reveals that raising cAMP via adenosine receptor A_{2A} is sufficient to inhibit IL-1β dependent JNK activity in HUVECs.

3.10.3.3 The role of PKA in the inhibitory effect of ATP on IL-1β mediated JNK signalling

Indirect inhibition of G_s dependent signalling can be achieved by utilising the pharmacological inhibitor of PKA, namely H89. Figure 3.30 showed that using both *in vitro* kinase assay and Western blotting show that pre-incubation of HUVECs with ATP (30 μM) reduced IL-1β mediated JNK signalling by approximately 50 %, which was reversed to 20 % in the presence of H89 (10 μM) (kinase assay: % IL-1β stimulation: ATP + IL-1β = 44.42 ± 5.32 %, H89 + ATP + IL-1β = 80.24 ± 5.14 %, $p < 0.001$) (Figure 3.30). The effect of H89 was concentration dependent, reversal was diminished at 3 and 1 μM of the compound. Nevertheless, H89 alone slightly inhibited the IL-1β dependent JNK activity (% IL-1β stimulation: 73.95 ± 7.73 %, $p < 0.05$), revealing the non-specific nature of the compound at high concentration.

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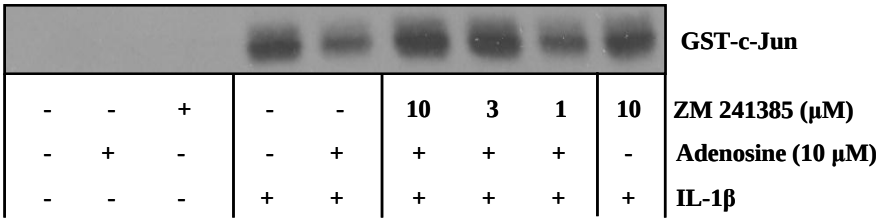

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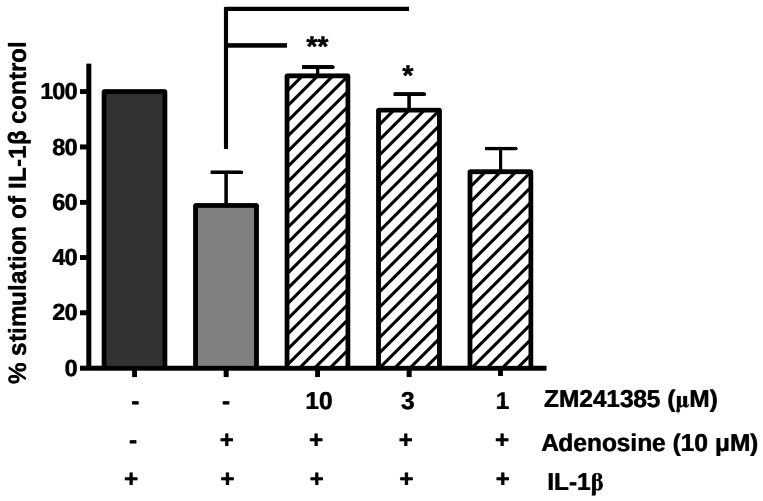

Figure 3.29: The role of A_{2A} receptor in the inhibitory action of adenosine on IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with increasing concentrations of ZM241385 (1 - 10μM) for 30 minutes, followed by adenosine (10 μM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in sections 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to combined adenosine and IL-1β stimulation. The statistical difference between IL-1β only and adenosine + IL-1β was not indicated in the graph.

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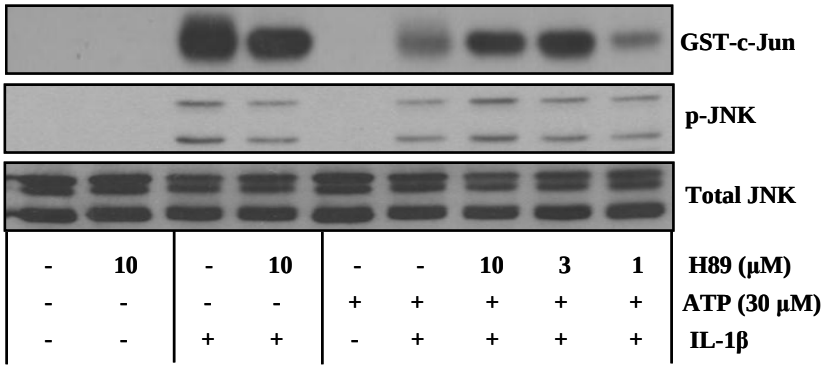

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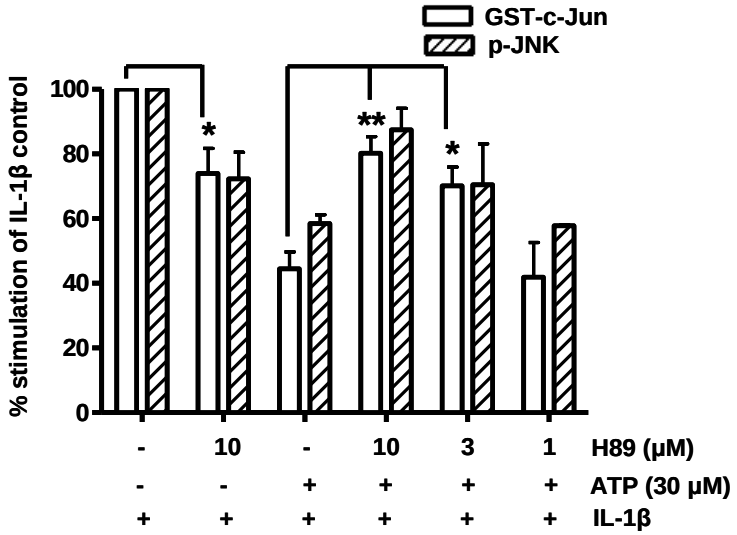

Figure 3.30: The role of protein kinase A in the inhibitory action of ATP in IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with increasing concentration of H89 (1 - 10μM) for 30 minutes, followed by ATP (30 μM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5 for kinase assay, n = 3 for Western blotting. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to the respective controls (GST-c-Jun phosphorylation) as indicated in the graph.

3.10.3.4 The role of EPAC in the inhibitory effect of ATP on IL-1β mediated JNK signalling

Increase in intracellular cAMP level is also linked to another signalling molecule, named exchange protein directly activated by cAMP (EPAC). However a specific pharmacological inhibitor for EPAC is unavailable to date. Thus, an EPAC activator, 8CPT-2Me-cAMP (Enserink et al., 2002) was applied as a pre-treatment for 30 minutes, followed by 30 minutes of IL-1β (Figure 3.31). This EPAC agonist only inhibited IL-1β induced JNK activity at high concentrations (30 μM = 25.3 ± 8.47 % inhibition), but this inhibitory effect was statistically insignificant. Thus, it could be concluded that ATP did not inhibit JNK signalling via EPAC.

3.10.4 The effect of combined $G_{q/11}$ and G_s blockade on ATP mediated inhibition of IL-1β mediated JNK signalling

As ATP mediated inhibition via both $G_{q/11}$ and G_s dependent pathways, the potential of synergism between G proteins in regulating IL-1β mediated JNK signalling was examined. As shown in Figure 3.32, pre-incubation of ATP (100 μM) inhibited IL-1β mediated JNK signalling by approximately 90 %. Pre-treatment with a maximum concentration of YM254890 only slightly reversed the inhibitory effect of ATP. Similarly a submaximal concentration of H89 promoted a small reversal of the effects of ATP. (% IL-1β stimulation: ATP + IL-1β = 6.86 ± 1.34 %, YM254890 + ATP + IL-1β = 22.05 ± 4.24 %, H89 + ATP + IL-1β = 17.35 ± 2.80 %, $p > 0.05$). However both agents in combination resulted in more than additive reversal of the inhibitory effects of ATP (% IL-1β stimulation: YM254890 + H89 + ATP + IL-1β = 66.99 ± 5.59 %, $p < 0.001$). These data suggested that stimulation of $P2Y_{11}$ can lead to both $G_{q/11}$ and G_s activation that functions co-operatively to inhibit IL-1β mediated JNK signalling.

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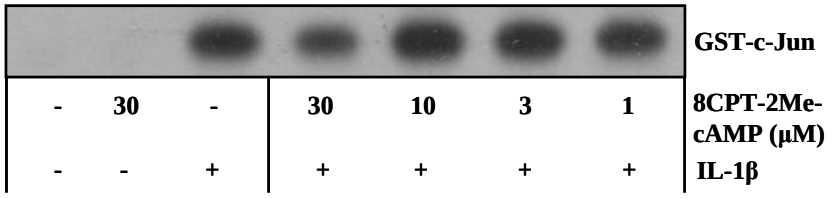

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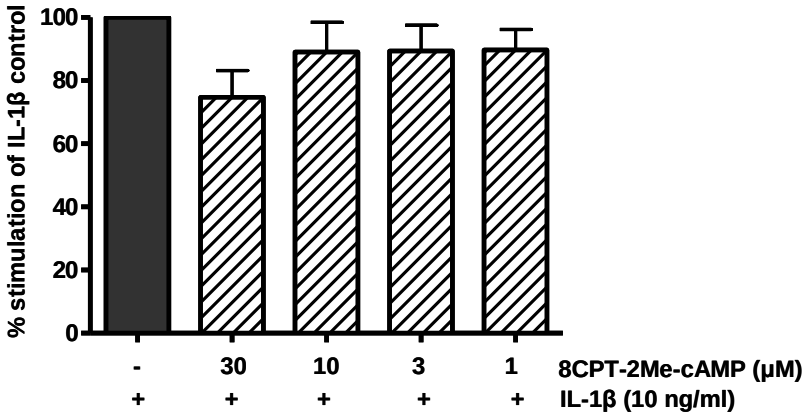

Figure 3.31: The role of EPAC in IL-1β mediated JNK activation in HUVECs

Confluent cells were incubated with increasing concentrations of 8CPT-2Me-cAMP (1 - 30 μM) for 30 minutes, followed by IL-1β (10 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no statistical difference was found between IL-1β only and 8CPT-2Me-cAMP + IL-1β.

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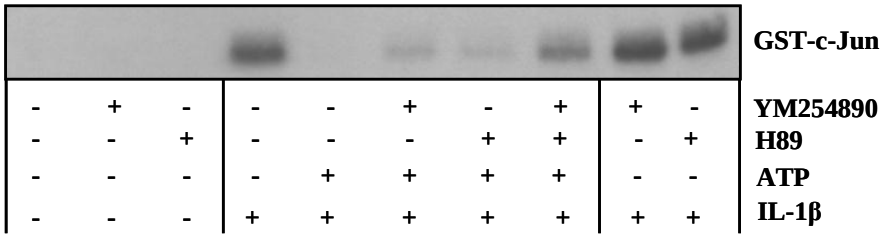

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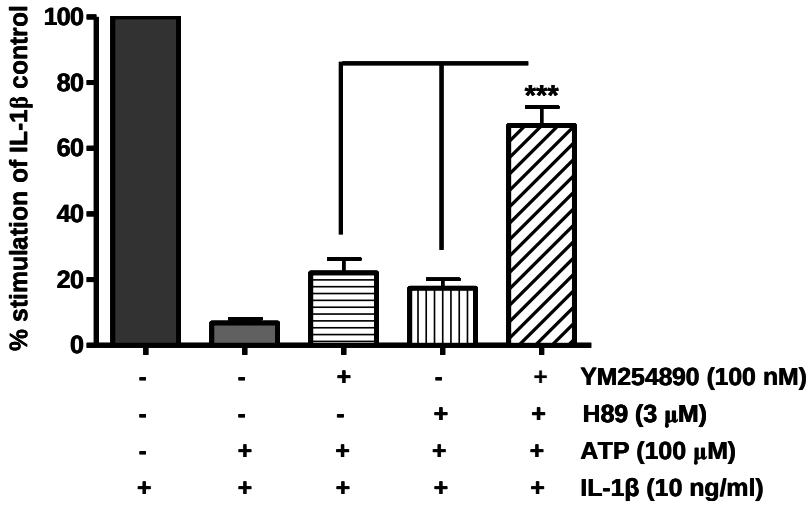

Figure 3.32: The role of combined $G_{q/11}$ and protein kinase A inhibition on ATP mediated inhibition of IL-1β driven JNK activation in HUVECs

Confluent cells were incubated with YM254890 (100 nM) and/or H89 (3 μM) for 30 minutes, followed by ATP (100 μM) for 30 minutes, and IL-1β (10 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in sections 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ for YM254890 + H89 + ATP + IL-1β compared to either YM254890 + ATP + IL-1β or H89 + ATP + IL-1β stimulation. The statistical difference between IL-1β only and other treatments were not indicated in the graph.

3.11 The effect of ATP on the pro-inflammatory proteins stimulated by IL-1β in HUVECs

A number of studies have shown that JNK activity is linked to the production of inflammatory proteins such as cyclo-oxygenase 2 (COX-2) and E-selectin (Min and Pober, 1997, Wu et al., 2006). The following section clarifies the significance of ATP mediated inhibition of JNK in reducing the expression of the pro-inflammatory protein, COX-2.

3.11.1 Characterisation of IL-1β mediated COX-2 production in HUVECs

The ability of ATP and IL-1β to induce COX-2 expression was monitored over an incubation period of 24 hours as shown in Figure 3.33. Stimulation of HUVECs with ATP (100 μM) alone showed no increase in COX-2 expression over 24 hours (data not shown). In contrast, IL-1β induced a strong, time-dependent increase in COX-2 expression which was manifest as early as 4 hours and maximal between 8 and 12 hours incubation (approximately 8.1 fold stimulation compared to unstimulated control at 8 hours). Subsequently, the concentration response curve for IL-1β mediated COX-2 expression was measured at the 12 hour time point as shown in Figure 3.35. Stimulation with IL-1β potently induced COX-2 expression in the low nM concentration range, reaching a peak between 3 – 5 ng/ml, with an EC_{50} value of approximately 0.6 ng/ml. Thus 5 ng/ml of IL-1β over with an incubation period of 12 hours was utilised for the following experiments.

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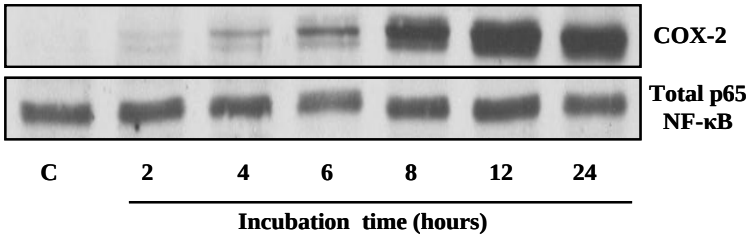

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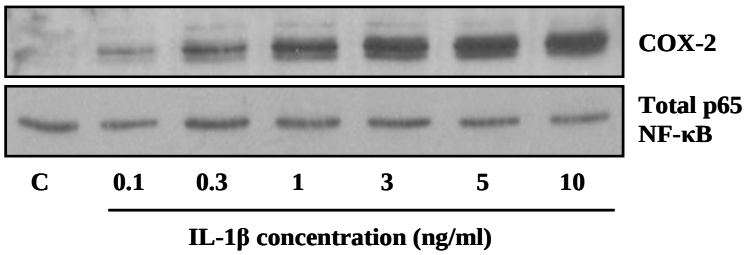

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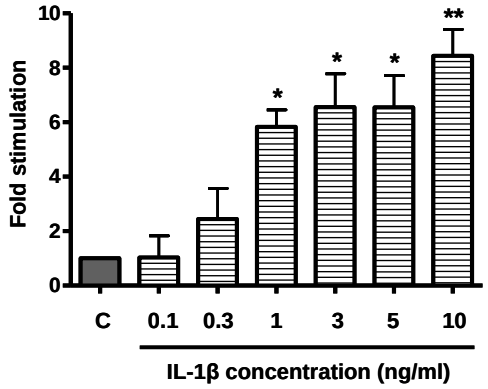

Figure 3.33: The kinetics and concentration dependent IL-1β production of COX-2 in HUVECs

Confluent cells were incubated with IL-1β (20 ng/ml) for the times indicated in (A) and at increasing concentrations (0.1 - 10 ng/ml) for 12 hours in (B). Expression of COX-2 was assessed by Western blotting as described in section 2.6. (A) and (B) showed representative blots and (C) showed the densitometric data for several independent experiments in (B), expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to unstimulated control.

3.11.2 Pre-incubation of ATP inhibited IL-1β mediated COX-2 production

Next, the effect of ATP pre-treatment on IL-1β stimulated inflammatory protein production was assessed. HUVECs were pre-incubated with ATP for 30 minutes, followed by stimulation with IL-1β for 12 hours (Figure 3.34). ATP exerted a concentration dependent inhibition of COX-2 expression, with approximately 50 % reduction achieved in response to 100 μM ATP (% IL-1β stimulation: 45.05 ± 4.39 %, $p < 0.001$). Combining the results from several experiments, an IC_{50} value of 19.44 ± 1.35 μM was obtained. In contrast, ATP did not reduce the expression of another inflammatory protein ICAM-1. Additional control experiments utilising the JNK inhibitor SP600125 (10 μM) and the IκB kinase inhibitor BMS-345541 (10 μM) confirmed that COX-2 expression was dependent on both JNK and NF-κB signalling whilst ICAM-1 and VCAM-1 expression were only dependent on the latter pathway. These data suggested that the inhibitory effect of ATP on COX-2 expression may be partly mediated through an inhibition of JNK signalling.

3.11.3 The effect of G protein inhibition in the inhibitory effect of ATP on IL-1β mediated COX-2 expression

Since the inhibition of $G_{q/11}$ and G_s dependent activity reversed the inhibitory effect of ATP on JNK activity, this section utilised both YM254890 and the H89 to elucidate the role of these mechanisms in the inhibitory effect of ATP in COX-2 expression mediated by IL-1β. Similar to the previous section, pre-incubation of HUVECs with ATP inhibited IL-1β induced COX-2 expression, but this effect was reversed by the pre-treatment with YM254890 (% IL-1β stimulation: ATP + IL-1β = 65.78 ± 3.99 %, YM254890 100 nM + ATP + IL-1β = 89.08 ± 4.28 %, $p < 0.01$) (Figure 3.35). Unfortunately, H89 alone abolished COX-2 and ICAM-1 expression, suggesting an unspecific effect of H89 in NF-κB. Collectively these results demonstrated that ATP mediated inhibition of JNK can be translated directly into the measurable physiological outcome in the form of JNK dependent COX-2 expression.

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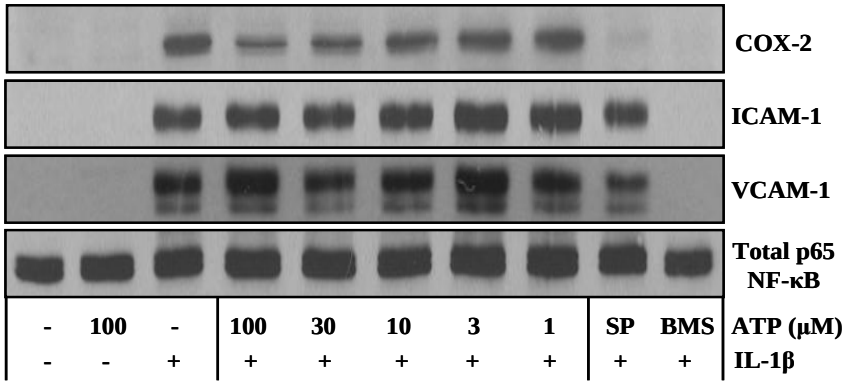

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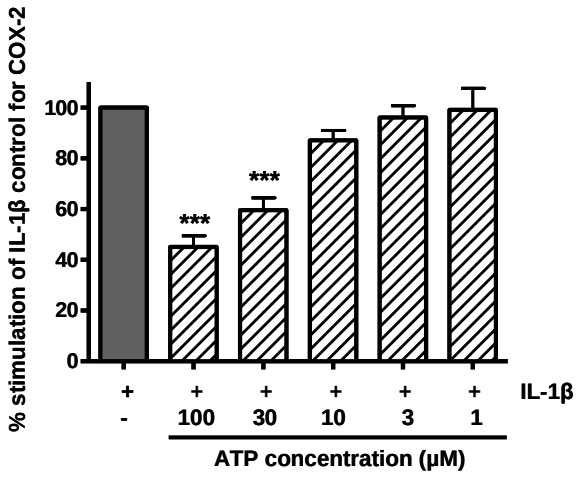

Figure 3.34: Concentration dependent ATP inhibition of IL-1β mediated COX-2 expression in HUVECs

Confluent cells were incubated with increasing concentrations of ATP (1 – 100 μM), JNK inhibitor SP600125 (SP) (10 μM) or NF-κB inhibitor BMS-345541 (BMS) (10 μM) for 30 minutes, followed by IL-1β stimulation (5 ng/ml) for 12 hours. Expression of COX-2 was assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments (COX-2), expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to IL-1β only stimulation.

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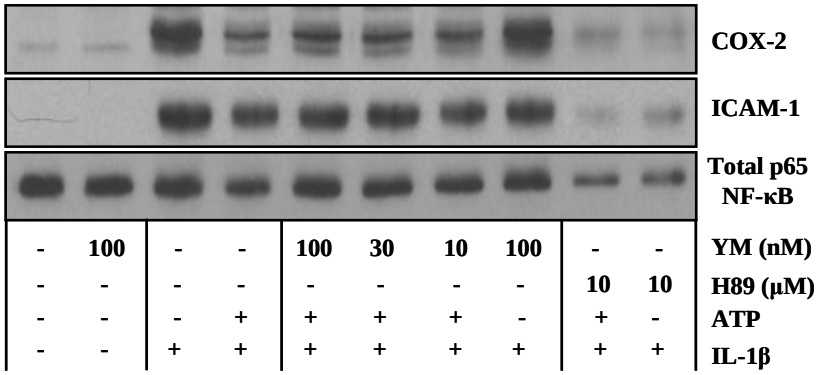

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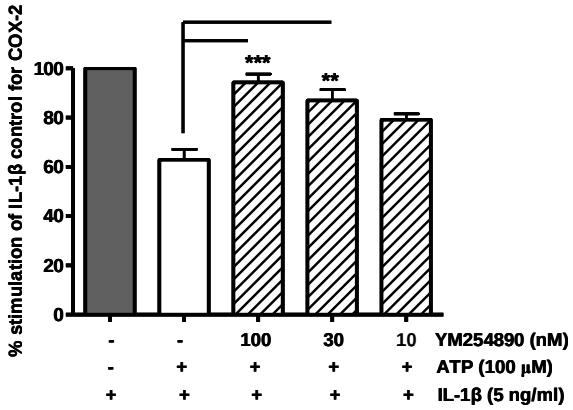

Figure 3.35: The role of $G_{q/11}$ in the inhibitory effect of ATP in IL-1β mediated COX-2 expression in HUVECs

Confluent cells were incubated with increasing concentrations of YM254890 (10 – 100 nM) for 15 minutes or H89 (10 μM) for 30 minutes, followed by ATP (100 μM) for 30 minutes and IL-1β (5 ng/ml) for a further 12 hours. Expression of COX-2 was assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments (COX-2), expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to combined ATP and IL-1β stimulation. The statistical difference between IL-1β only and ATP + IL-1β was not indicated in the graph.

3.11.4 The effect of adenosine on IL-1β mediated COX-2 expression

As adenosine also inhibited IL-1β mediated JNK signalling, the effect of pre-incubation of adenosine on IL-1β mediated COX-2 production was measured as shown in Figure 3.36. The inhibition was concentration dependent with a maximum reduction of approximately 25 % at 100 μM (% IL-1β stimulation: 75.36 ± 10.12 %). However even at this high concentration, inhibition did not reach statistical significance ($p > 0.05$).

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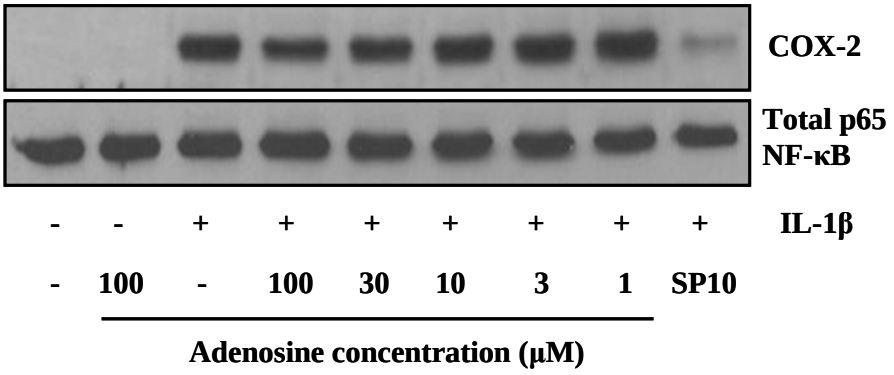

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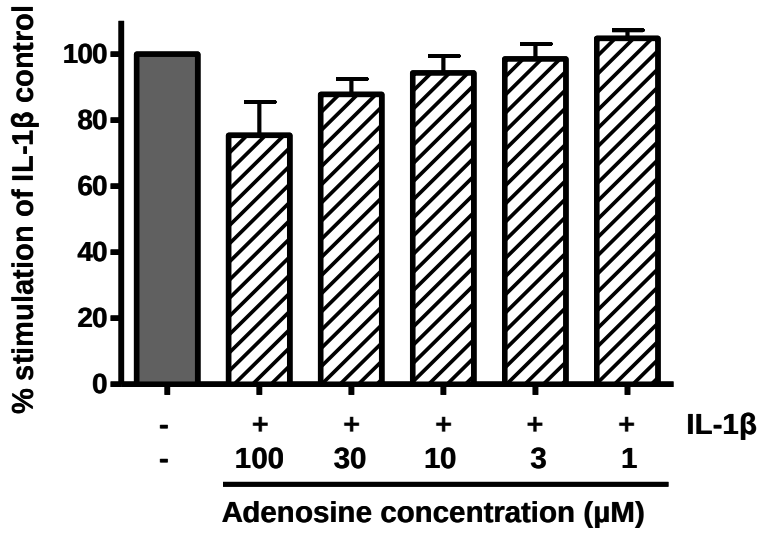


Figure 3.36: Concentration dependent adenosine inhibition of IL-1β mediated COX-2 expression in HUVECs

Confluent cells were incubated with increasing concentrations of adenosine (1 – 100 μM) or SP600125 (SP10) (10 μM) for 30 minutes, followed by IL-1β stimulation (5 ng/ml) for 12 hours. Expression of COX-2 was assessed by Western blotting as described in section 2.6. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no difference was found between IL-1β and adenosine + IL-1β.

3.12 Discussion

In this chapter, it was hypothesised that pre-activation of GPCRs could protect HUVECs from the damaging effects of pro-inflammatory mediators induced by cytokines, such as TNF-α and IL-1β. Pre-incubation of HUVECs with ATP was observed to activate the $P2Y_{11}$ receptor which then inhibited cytokine-induced JNK signalling. Pre-treatment with the $G_{q/11}$ inhibitor, YM254890, revealed that $G_{q/11}$ plays a significant, but not an absolute role in the inhibitory effect of purinergic receptor activation. Incubation of HUVECs with adenosine that increases intracellular cAMP concentration also produced a concentration dependent inhibition in IL-1β mediated JNK activity, suggesting that signalling components downstream of G_s can participate in this inhibitory pathway. The role of G_s was also confirmed as PKA inhibitor H89 also reversed the inhibitory effect of ATP. The inhibitory effect of ATP upon JNK signalling also translated into a reduction in the expression of the pro-inflammatory protein, COX-2. As a summary, these reveal a novel anti-inflammatory mechanism of GPCRs, for $P2Y_{11}$ and A_{2A} receptors, thereby switching off a key SAPK signalling involved in inflammation in a cardiovascular setting.

Although these experiments were performed *in vitro*, these results may be related to a physiological setting. For example, a number of studies have demonstrated that shear stress induces the release of ATP from the endothelial cells and erythrocytes (Milner, Ralevic et al. 1989; Milner, Bodin et al. 1990; Sprung, Sprague et al. 2002; Moehlenbrock, Price et al. 2006). Platelets also possess vesicles which contain ATP (approximately 40 nM of ATP and ADP per mg of protein) which could be released upon degranulation to increase the local ATP concentration (Gordon, 1986). A higher ATP concentration is also detected in hypoxia (Bodin and Burnstock, 1995) and inflammatory conditions (Bodin and Burnstock, 1998). Furthermore, although clinical human plasma samples reported an average ATP concentration of 1 μM (Gorman et al., 2007), the local ATP concentration at the endothelial cell surface could be higher during injury with a maximal of 14 μM detected in the blood by a luciferin-luciferase method (Born and Kratzer, 1984). These results are supported by another *in vitro* study where 12 μM of ATP was detected in blood after platelet

activation (Gordon, 1986). These concentrations are in keeping with those required to inhibit JNK signalling, which are similar to the concentration used to stimulate intracellular Ca^{2+} release, inositol phosphate accumulation and phosphorylation of ERK (Montiel et al., 2006, Patel et al., 1996, Short et al., 2000, Xiao et al., 2011).

A similar issue was also involved in the concentration of cytokines used in this study in order to generate a measurable JNK signal. This was in the 1- 20 ng/ml range and whilst this is consistent with the concentrations used in a number of studies both within the laboratory and elsewhere (Karmann et al., 1996, Mong et al., 2008, Read et al., 1997, Wadgaonkar et al., 2004, McIntosh et al., 2010, Paul et al., 2000), these concentrations are much higher than those observed *in vivo*. Numerous studies have shown that the serum concentration of cytokines are within the pg/ml range, at approximately 25 pg/ml and < 1 pg/ml for TNF-α and IL-1β respectively for healthy individuals (Di Iorio et al., 2003, Saito et al., 1999b). Even in disease states, such as diabetes and coronary heart disease, only slight elevation in serum cytokine levels are detected, for instance, congestive heart failure and angina patients measure an average of 1.39 pg/ml and 18.8 pg/ml in IL-1β levels (Di Iorio et al., 2003, Doganay et al., 2002, Hasdai et al., 1996). The pro-inflammatory cytokines, TNF-α and IL-1β, are secreted mainly by macrophages and mononuclear cells that accumulate in the fatty streaks of the atherosclerosis lesion (Kishikawa et al., 1993, Tipping and Hancock, 1993). Endothelial cells could also produce these cytokines in response to pro-inflammatory stimuli (as discussed in the introduction chapter). Thus both ATP and cytokines are present within close vicinity, suggesting that the inhibitory effect of ATP observed in this study could be observed under physiological conditions. In retrospect, the cytokine-ATP ratio could have been more appropriately set, however in some experiments in this study (section 3.6.3) lower concentrations of IL-1β were used which demonstrated a similar level of inhibition.

Given the limitations of using an *in vitro* cell model and using relatively high concentrations of ATP and cytokines, it was essential to establish the validity of the

inhibitory effect of ATP. Initial studies whilst demonstrating that ATP could inhibit the JNK signalling mediated by both TNF-α and IL-1β, also showed that incubation of HUVECs with ATP alone generated a submaximal JNK signal, particularly at high concentrations. This concentration-dependent effect raised the possibility that the inhibition mediated by ATP was merely due to a depletion of the cellular JNK pool by prior GPCR activation, limiting the amount of JNK available for subsequent activation by cytokines. A similar phenomena was also observed in $P2Y_2$ stimulated EAhy926 cells and following activation of PAR_2 in NCTC2544 cells (Kanke et al., 2001, McIntosh et al., 2010, Paul et al., 2000). In another study histamine was found to initially stimulate p38 MAPK and JNK activity via the H_1 receptor in HUVECs, and then reduce the subsequent activation of these pathways by either TNF-α or thrombin (Steffel et al., 2006). Since both p38 MAPK and JNK signalling were inhibited by histamine, this suggests inhibition at a point upstream in the pathway, rather than a depletion of JNK pools. In addition, other GPCRs were also found to inhibit cytokine mediated JNK activity without prior activation of MAPK, as observed for CB_2 inhibition of TNF-α induced MAPK signalling in human coronary artery smooth muscle cells (Rajesh et al., 2008). In this chapter, the inhibitory effect of adenosine also occurred without prior activation of JNK. Overall, these data demonstrated that prior activation of JNK signalling is not a prerequisite for subsequent inhibition.

The inhibitory effect of ATP shown in this study also extends to two different cytokines, TNF-α and IL-1β, which utilise different adaptor proteins for downstream signalling. In addition, activation of the H_1 receptor was found to inhibit JNK signalling mediated by both the cytokine receptor, TNF-R1 and the GPCR, PAR_1 (Steffel et al., 2006). These results further imply that GPCR activation may result in the inhibition of common signalling intermediates of JNK, possibly MKK7 or further upstream. In order to clarify the specificity of JNK inhibition by ATP, sorbitol and anisomycin were used. These classical activators of SAPK have been previously demonstrated to activate JNK signalling in Hela, U937 monocytes and rat ventricular myocytes (Bogoyevitch et al., 1995, Moriguchi et al., 1997, Raingeaud et al., 1995).

Both these JNK activators were minimally affected by ATP pre-incubation (Section 3.8), again suggesting that the effect of P2Y receptor is specific to cytokine mediated JNK signalling only. However there is a caveat in that JNK signalling stimulated in response to cytokines may differ from activation by sorbitol and anisomycin. Although the cytokine TNF-α mainly requires upstream MKK7 for JNK signalling (Moriguchi et al., 1997), in contrast, sorbitol and anisomycin were found to utilise both MKK7 and MKK4 for JNK activation (Moriguchi et al., 1997). It is possible that the effect of ATP lies upstream of MKK7 and that activation of MKK4 by anisomycin or sorbitol could compensate for the inhibition of MKK7. However, there is no evidence currently available to support this hypothesis.

A further criteria in establishing the specific nature of the inhibitory effect of ATP on JNK signalling, are the effects on other signalling pathways. ATP had little effect on p38 MAPK and NF-κB activation in response to TNF-α and IL-1β. This again supports the idea that ATP is acting specifically on a level upstream in the JNK signalling pathway, at a point of bifurcation. However these results contradicted with the inhibitory effect of $P2Y_2$, PAR_2 and H_1 receptors on TNF-α mediated p38 MAPK and/or NF-κB signalling (McIntosh et al., 2010, Paul et al., 2000, Steffel et al., 2006). This could be due to differences in the site of inhibition within the cytokine pathway; whilst PAR_2 receptor prevented the recruitment of TRADD and RIP1 to TNF-R1, H_1 receptor induced the shedding of TNF-R1 from the cell surface and mobilised TNF-R1 from the Golgi apparatus (McIntosh et al., 2010, Wang et al., 2003). Thus, the inhibition mechanism of $P2Y_{11}$ receptor may differ from PAR_2 and H_1 receptors.

A number of approaches were used to better define the receptors involved in the effect of ATP inhibition. HUVECs abundantly express $P2Y_{11}$, $P2Y_1$ and $P2Y_2$ receptors as demonstrated using RT-PCR (Wang et al., 2002, Xiao et al., 2011). Use of receptor antagonists confirmed that $P2Y_{11}$ receptor transduced the downstream inhibitory effect of ATP in HUVECs. The results in this chapter also exclude a role for $P2Y_1$ receptor, this is important as a previous study has shown that $P2Y_{11}$

receptor internalisation requires the hetero-oligomerisation with $P2Y_1$ receptor (Ecke et al., 2008). This suggests that $P2Y_{11}$ receptor internalisation is not a prerequisite for inhibition of JNK signalling, ruling out a role for a β-arrestin dependent mechanism (Hoffmann et al., 2008). Even though the functional role of internalisation in the actions of $P2Y_{11}$ receptor has not been studied, other GPCRs such as PAR_2, β_2-adrenoceptor and angiotensin II receptors have been found to couple to β-arrestin to prolong ERK signalling (Defea, 2008, DeFea et al., 2000). Thus rapid signalling events, such as transient ERK activation and inositol phosphate (IP_3) formation, that are G-protein dependent, are still intact and could mediate the inhibitory effects of ATP observed in this study.

Furthermore, studies using UTP has shown that it did not affect IL-1β driven JNK activity, thus excluded a role for $P2Y_2$ receptor in mediating the inhibitory effect of ATP. This observation was different from an earlier study whereby $P2Y_2$ receptor was shown to reduce TNF-α mediated JNK signalling in EAhy926 and NCTC2544 cells (Paul et al., 2000). In the same study, it was found that although 1321N1 human astrocytoma also expressed $P2Y_2$ or $P2Y_4$ receptor, in this instance UTP activation did not produce the same inhibitory effect as observed in the other two cell lines. Thus, different receptor expression level of GPCRs in different cell lines may play a key role in influencing cytokine mediated signalling, suggesting that the $P2Y_2$ receptor expression level may be too low to transduce the inhibitory signal of UTP in HUVECs as observed in this study.

In the physiological environment, ATP is prone to undergo degradation by various ecto-nucleotidases. Endothelial cells express abundant NTPDase1 and ecto-5'-nucleotidase compared to other cell types, producing high concentrations of metabolites such as ADP, AMP and adenosine (Wood et al., 2002). Shear stress also induces the release of soluble ecto-ATPase along with ATP secretion (Yegutkin et al., 2000), increasing the local concentrations of ATP and their metabolites on the endothelial cell surface. A lower degree of $P2Y_1$ receptor activation by ADP could

also occur as extracellular ecto-nucleotide kinases could convert ATP to ADP (approximately 25 % of ADP formed from ATP) within the stimulation time frame of 30 minutes (Yegutkin et al., 2001). Pre-treatment with the ecto-nucleotidase inhibitor ARL67156 did not increase the reversal of inhibitory effect of ATP, excluding this possibility and confirming that ATP itself inhibited IL-1β mediated JNK signalling via $P2Y_{11}$ receptor.

It should be noted however, that whilst adenosine was not responsible for the inhibitory action of ATP, it was nevertheless shown to potently inhibit JNK signalling over the micromolar concentration range. This may again be related to physiological or pathophysiological conditions. The exact plasma concentration of adenosine is not easily determined as adenosine has a short plasma half-life (1.5 seconds) due to rapid uptake and metabolism (Moser et al., 1989). Thus depending on the design of study, huge discrepancies in plasma adenosine concentrations have been recorded. For example, approximately 51 nM was detected in volunteers exposed to moderate hypoxia (Saito et al., 1999a), but in another study, 180 nM of plasma concentration was measured in non-pregnant women (Yoneyama et al., 2000). Nevertheless the local adenosine concentration on the endothelial cell surface could be within the micromolar range due to the extracellular conversion from ATP. In this study, inhibition was mediated via the A_{2A} receptor, as pre-treatment with ZM241385 completely reversed the inhibitory effect of adenosine. Several studies using RT-PCR have shown strong expression of both A_{2A} and/or A_{2B} receptor in HUVECs (Fang and Olah, 2007, Feoktistov et al., 2002, Montesinos et al., 1997). Other studies investigating the effect of adenosine incubation in HUVECs have revealed that 10 µM could stimulate nitric oxide and tissue factor expression via intracellular cAMP and cGMP signalling (Deguchi et al., 1998, Gabazza et al., 2002). Thus, the concentration range of adenosine applied in this study is justified in accordance with the activation of adenosine receptors in numerous studies performed *in vitro* and could suggest a dual inhibitory mechanism involving both purines and pyrimidines.

In the second part of the chapter, the role of G protein linked signalling pathways in the inhibitory effect of ATP on cytokine mediated JNK signalling was studied. The $P2Y_{11}$ receptor has been demonstrated to couple to both $G_{q/11}$ and/or G_s (Communi et al., 1999) therefore both pathways could be involved. In order to investigate the role of $G_{q/11}$ in the inhibitory effect of ATP, a specific $G_{q/11}$ inhibitor, YM254890, was employed. This compound has been shown to exhibit a high potency for $G_{q/11}$, inhibiting [^{32}S]GTP binding and [^{3}H]GDP dissociation from $G_{q/11}$ with an IC_{50} between 10^{-7} to 10^{-8} M (Nishimura et al., 2010, Takasaki et al., 2004). YM254890 has been previously characterised in various *in vitro* cell lines and *ex vivo* mouse platelets (Uemura et al., 2006b), however the concentration range used varied greatly depending on agonist concentration, cell type and endpoints measured. Lower nanomolar concentrations (up to 100 nM) were shown to inhibit $G_{q/11}$ mediated Ca^{2+} signalling in rat gliomas (Takasaki et al., 2004), fibroblasts (Orth et al., 2005) and platelets (Kim and Kunapuli, 2011, Uemura et al., 2006a), whilst other studies have used 1 - 10 μM to inhibit thrombin mediated JNK signalling in endothelial cells (Miho et al., 2005) and actin depolymerisation by ATP in keratinocytes (Taboubi et al., 2007). In this current study, the ability of YM254890 to partially reverse the inhibitory effect of ATP at 100 nM, however the concentration of YM254890 required to inhibit the $P2Y_{11}$ mediated IP_3 accumulation in HUVECs was not established. Only one study in rat cardiac fibroblasts has shown that pre-treatment of YM254890 at 1 μM completely reversed IP_3 accumulation and cAMP increase for the incubation of 100 μM of UTP and UDP (Talasila et al., 2009). Results from numerous $G_{q/11}$-protein coupled receptors (H_1 and PAR_2) have also supported the role of $G_{q/11}$ in inhibiting cytokine mediated signalling (McIntosh et al., 2010, Paul et al., 2000, Wang et al., 2003).

Next, the role of PKC activation in the regulation of cytokine mediated JNK signalling downstream of $G_{q/11}$ was studied. The expression of multiple PKC isoforms in HUVECs, including α, β, ζ δ and ε, has been demonstrated previously (Dang et al., 2004, Ross and Joyner, 1997, Shizukuda et al., 1999), several of which have also been shown to be activated through GPCRs (Montiel et al., 2006, Short et

al., 2000). It was expected that PMA in keeping with previous results (Byun et al., 2006, Paul et al., 2000) would cause an inhibitory effect. However in preliminary experiments, it was found that PMA at 100 nM stimulated a strong and persistent JNK signal over 90 minutes and the activity was potentiated when in combination with TNF-α or IL-1β (results not shown). These results made it difficult to interpret and prevented any long term downregulation studies. Furthermore, pre-treatment with the broad spectrum PKC inhibitors GF109203X and Go6976 failed to reverse the inhibitory effect of ATP. As these compounds have been shown to inhibit primarily the conventional PKC isoforms (α and β) (Martiny-Baron et al., 1993, Toullec et al., 1991), these data suggested that neither of these isoforms played a role in the regulatory effect of ATP, even though PKCα is one of the most abundantly expressed PKC isoforms in HUVECs (Ross and Joyner, 1997, Shizukuda et al., 1999). Novel and atypical PKCs ε, δ and ζ are also inhibited by high GF109203X concentrations (Toullec et al., 1991), suggesting that these PKC isoforms are also not involved. Given that YM254890 caused only a partial reversal of the cytokine mediated JNK signalling, it is possible that the effect of ATP involves a calcium-dependent, but PKC-independent action.

It should be noted that these results contrast with other studies which demonstrated a role for PKC isoforms in the regulation of cytokine mediated JNK signalling. PKC ζ was found to mediate the inhibitory effect of laminar flow in TNF-α induced JNK signalling in HUVECs (Garin et al., 2007). On the other hand, in prostate cancer cells, PKC ε siRNA augmented the JNK and p38 MAPK phosphorylation in response to TNF-α (Meshki et al., 2010). Thus siRNA for PKC isoforms including PKC ζ and ε could be applied in future studies to examine its role in mediating the inhibitory effect of ATP via $P2Y_{11}$ receptor.

Incomplete reversal of the inhibitory effect of ATP by YM254890 suggested that other signalling pathways may also contribute. In this chapter, the ability of G_s to modulate JNK signalling was demonstrated indirectly via adenosine mediated

inhibition of IL-1β signalling. The downstream signalling of G_s is closely linked to an increase in intracellular cAMP level, followed by either PKA or EPAC activation. Numerous studies have revealed that cAMP alone could either inhibit or activate JNK signalling, depending on the cell type studied. An increase in intracellular cAMP level in T lymphocytes (Hsueh and Lai, 1995), gingival and lung fibroblasts (Black et al., 2007), lymphoblastic leukaemia cells (Miller et al., 2007) and cardiac muscle cells (Chae et al., 2004) was shown to inhibit JNK activity but the reverse was observed in hamster smooth muscle cells (Harada et al., 1999, Yamauchi et al., 2001a, Zhang et al., 2004) and hepatocytes (Zhang et al. 2004). Collectively these studies suggest that JNK can be regulated by cellular cAMP levels.

Extending the observations in these studies, a role for PKA in the inhibitory effect of ATP was examined and confirmed by the pre-treatment of H89. However, this widely used PKA inhibitor has several non-specific effects, such as inhibition of MSK1, S6K1 and ROCK-II kinase, achieved with similar or greater potency to PKA itself (Davies et al., 2000). In the context of this study, the non-specific effects of H89 could account for the slight reduction in JNK activity seen for combined H89 and IL-1β stimulation. Further studies using PKA siRNA could then confirm the results of H89. Furthermore, partial inhibition of both $G_{q/11}$ and G_s produced an additive reversal effect in ATP mediated JNK signalling, suggesting that these G proteins dependent pathways have the potential to work in concert.

The role of EPAC which lies downstream of cAMP was also investigated. There are two isoforms of EPAC, but only EPAC1 is present in HUVECs as shown by RT-PCR (Fang and Olah, 2007). Due to the current lack of a potent pharmacological inhibitor of EPAC, the EPAC activator, 8CPT-2Me-cAMP was used instead. This cAMP analogue is highly specific for EPAC, with 10-fold higher affinity against PKA (Enserink et al., 2002). However, in this chapter this compound was found to have no inhibitory effect on cytokine mediated JNK activity at 30 μM. In contrast, the majority of the EPAC studies in HUVECs have used higher micromolar

concentrations (50 - 200 µM) to activate ERK signalling, increase cell adhesion to monocytes and inhibit thrombin mediated cell permeability (Aslam et al., 2010, Chen et al., 2011, Fang and Olah, 2007). Thus experiments using increasing concentration of the EPAC activator could have been used. However in another study using human airway smooth muscle cells, 8CPT-2Me-cAMP inhibited epidermal growth factor induced proliferation at a lower concentration (IC_{50} = 1.6 µM) (Kassel et al., 2008) whilst 20 µM was also shown to be sufficient to phosphorylate ERK, Akt, eNOS and CREB in HUVECs (Namkoong et al., 2009).

In the last part of this chapter, the relevance of ATP mediated inhibition of JNK signalling was assessed by examining JNK-dependent COX-2 expression in HUVECs. COX-2 generates the production of pro-inflammatory prostaglandins, such as PGE_2, prostacyclin, thromboxane and isoprostanes which are elevated in atherosclerosis (Belton et al., 2000). Basal levels of COX-2 in endothelial cells are normally low, but is highly inducible by cytokines (lipopolysaccharides, TNF-α and IL-1β) and epidermal growth factor (Akarasereenont et al., 1995, Maier et al., 1990). Higher mRNA levels and expression staining was observed in atherosclerotic areas, relative to non-disease segments (Belton et al., 2000, Cipollone et al., 2005a), supporting the role of COX-2 in the pathogenesis of atherosclerosis. Increased COX-2 led to PGE_2 production that could activate the over-expressed EP_4 receptors in the plaque (Cipollone et al., 2005b), which then caused an increase in metalloproteinases (MMP) 2 and 9 production, to stimulate plaque instability. Furthermore, JNK signalling has been implicated in COX-2 production in various cells, such as macrophages, endothelial cells and chondrocytes (Nieminen et al., 2006, Nieminen et al., 2005, Wu et al., 2006).

Pre-incubation of ATP produced a concentration dependent reduction in COX-2 expression, as did the JNK inhibitor, SP600125. This suggested that ATP may mediate its effect on COX-2 expression by inhibiting JNK signalling. Studies examining the COX-2 gene promoter reveal the presence of AP-2, SP-1, NF-IL6,

NF-κB, CRE, C/EBP and Ets-1 transcription factor binding sites (Appleby et al., 1994, Tazawa et al., 1994). JNK mediated activation of c-Jun is reported to bind to the CRE/E-box sequence of the COX-2 promoter in HUVECs to increase COX-2 expression (Schroer et al., 2002). COX-2 expression is also sensitive to inhibition by the IKK inhibitor BMS-345541, confirming a role for NF-κB in the regulation of this enzyme. ATP did not affect IL-1 beta induced NF-κB signalling and its inhibitory effect is therefore not mediated by this mechanism, however ATP could act synergistically with IKK inhibition. In order to confirm the anti-inflammatory role of ATP, other JNK dependent genes with an AP-1 binding site at the promoter region such as E-selectin and MMP9 expression could be measured via gelatin zymograph and ELISA.

In addition to reversing the inhibitory effect of ATP on IL-1β mediated JNK signalling, YM254890 also partially reversed the inhibition of COX-2 expression. This again suggests that ATP mediated inhibition of JNK has a functional outcome. However the results of H89 could not be interpreted appropriately as H89 alone effectively reduced IL-1β mediated COX-2 expression. As mentioned earlier, H89 can also inhibit MSK1, which phosphorylates p65 NF-κB (Reber et al., 2009), thus decreasing NF-κB activity at the COX-2 transcription site. In the future, other approaches including siRNA could clarify the role of G_s in COX-2 production induced by IL-1β. On the other hand, the reduction in COX-2 expression by adenosine is minimal, suggesting that increasing intracellular cAMP and activating PKA alone is not sufficient to inhibit COX-2 expression. Further experiments are required to determine any possible difference in the kinetics of inhibition that could cause these differences in results.

Finally, the inhibitory effect of ATP and adenosine on cytokine mediated JNK signalling could be related to the physiological, anti-inflammation traits of laminar shear stress. Several studies have indicated that laminar shear stress could inhibit TNF-α mediated JNK signalling via several mechanisms. Laminar flow activates

MEK5 and ERK5, which in turn inhibits TNF-α mediated JNK signalling to reduce VCAM-1 expression in endothelial cells (Surapisitchat et al., 2001, Li et al., 2008). Further studies show that this effect could be mediated by KLF2 (Boon et al., 2010), since a dominant negative ERK5 kinase, MEK5, reduces kruppel-like factor 2 (KLF2) induction by flow (Parmar et al., 2006). Another *ex vivo* study in rabbit aorta demonstrated a similar reduction in TNF-α induced VCAM-1 expression following pre-exposure to normal shear stress, which correlates with inhibition of TNF-α mediated MAPK signalling through disruption of the TNFR1-TRAF2 complex formation (Yamawaki et al., 2003). It is possible that ATP might mimic some of the effects of laminar shear stress although the effect of ATP is far more rapid in onset. Nevertheless future studies could investigate the role of some of the intermediates discussed above.

In summary, the activation of the P2Y receptors by ATP confers an anti-inflammatory role in endothelial cells through the inhibition of cytokine mediated JNK signalling. Although ATP alone generated a small and transient JNK signal, the results suggest it could reduce the detrimental JNK activity induced by pro-inflammatory cytokines. This study also revealed for the first time that both $G_{q/11}$ and G_s protein dependent events can activate different signalling pathways to produce similar inhibitory effects on IL-1β mediated JNK signalling. The findings in this chapter are summarised in Figure 3.37.

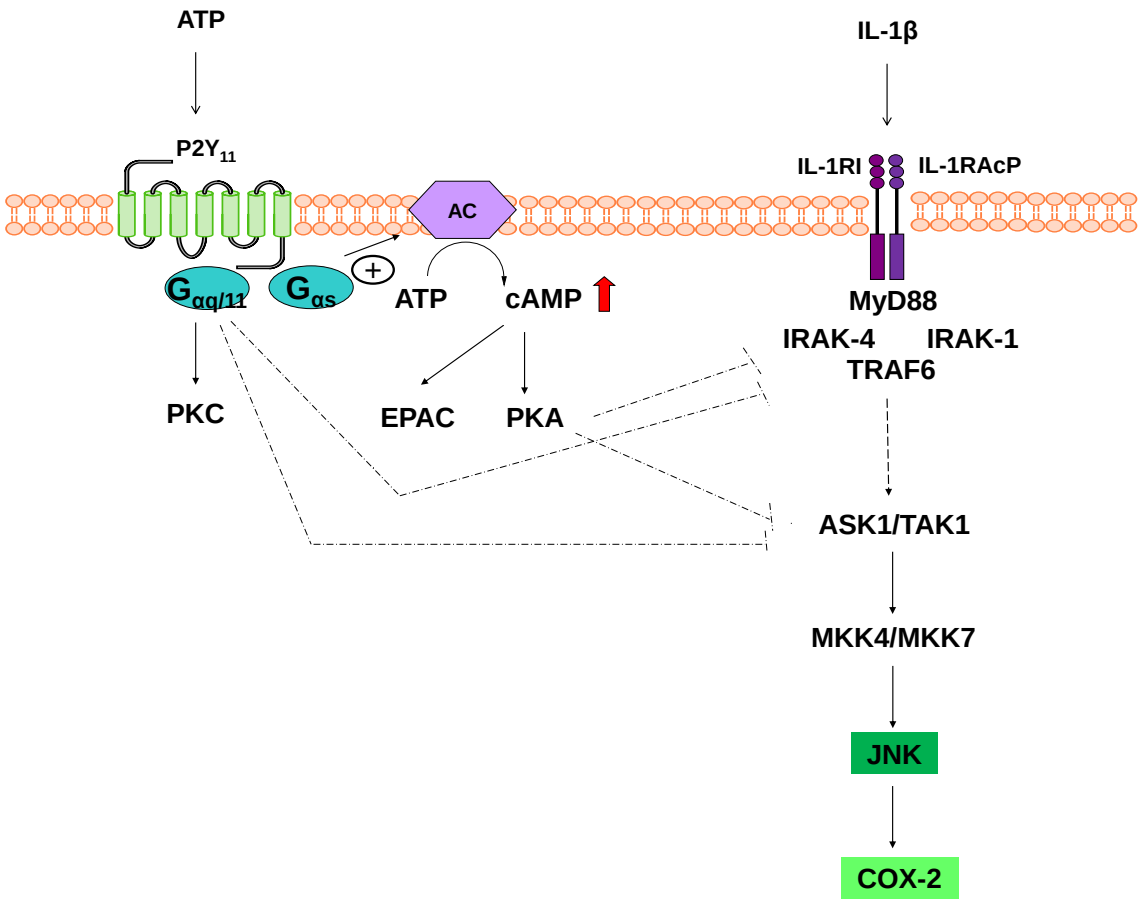


Figure 3.37: Current working model of GPCR signalling inhibition

A simplified figure on the signalling components involved in the inhibition properties of $P2Y_{11}$ receptor towards IL-1β mediated JNK signalling. However, the exact components that are inhibited in the JNK signalling cascade are yet to be identified. The dash-dot lines indicated the possible inhibitory point for ATP.

4.0 The inhibitory effect of P2Y receptor in cytokine- and UVC-mediated JNK activity in cancer cells

4.1 Introduction

Having established the potential of purinoceptor mediated inhibition of cytokine stimulated JNK signalling in endothelial cells, this chapter then investigated this inhibitory effect in other cell types. Thus, the inhibitory effect of ATP and adenosine was studied in both breast cancer cells and chondrosarcoma cells. Due to the high abundance of ATP in the cancer cell environment, activation of purinoceptors could play an important role in cancer progression either via its downstream signal activation or its interaction with other signalling pathways. Elevated concentrations of ATP in cancer was first demonstrated clearly in an *in vivo* mouse model using a luciferase reporter system following injection with melanoma MZ2-MEL cells (Pellegatti et al., 2008). Bioluminescence measurements demonstrated up to 100 µM ATP at the tumour site, but no ATP was observed in normal tissues. Furthermore, comparison of ATP secretion into the medium of different breast cancer cells *in vitro*, showed that phenotypically aggressive cancer cells (MDA-MB-231) produced approximately 10-fold higher ATP concentration in contrast to the less aggressive cancer cells (MCF7) (Kawai et al., 2008).

Whilst JNK activity is stimulated by cytokines, several other agents have been shown to activate JNK signalling in cancer cells, including UVC, DNA damaging agents and microtubule-interfering agents (Osborn and Chambers, 1996, Saleem et al., 1995, Sanchez-Perez et al., 1998, Wang et al., 1998b, Chen et al., 1996b). Furthermore, the role of JNK in the apoptotic effect of UVC has been directly demonstrated in various cell lines, including human fibroblasts, Jurkat T cells and human embryonic kidney cells (Wu et al., 2002, Hamdi et al., 2005, Chen et al., 1996b), but this phenomenon has not been shown in cancer cells. Activation of JNK signalling also plays an important role in cell cycle progression via phosphorylation of mitosis checkpoint proteins cdc25C and Cdh1 (Gutierrez et al., 2010, Gutierrez et al., 2010(a)), suggesting variability in the role of JNK in cellular survival. Specifically for breast

cancer, deficiency in JNK1/2 has been found to increase tumour formation (Cellurale et al., 2012). Thus, breast cancer cells may be a good model to understand the role of JNK in apoptosis in cancer.

In this chapter, the ability of ATP and adenosine to inhibit cytokine mediated JNK signalling was examined in the breast cancer cell line, MDA-MB-231, and the chondrosarcoma, SW1353. The inhibitory effect of ATP upon UVC dependent JNK signalling was also studied. Surprisingly, although the kinetics and magnitude of JNK activation induced by UVC differs greatly from cytokines, ATP also inhibited JNK signalling induced by UVC. The role of JNK inhibition in MDA-MB-231 was then investigated in relation to apoptosis.

4.2 Characterisation of the inhibitory effect of ATP and adenosine in cytokine mediated JNK signalling in MDA-MB-231

4.2.1 ATP and adenosine mediated inhibition of TNF-α induced JNK signalling in MDA-MB-231

Initial studies in MDA-MB-231 focused on determining the expression of P2Y and TNF-R1 on the cell surface by measuring the MAPK activity in the presence of ATP (100 μM) and TNF-α (10 ng/ml). As demonstrated in Figure 4.1A, ATP alone activated neither JNK nor ERK signalling as measured by *in vitro* kinase assay and Western blotting over 60 minutes. Therefore, the presence of purinergic receptors in MDA-MB-231 could not be confirmed via monitoring of MAPK activity. Nevertheless, the presence of TNF-R1 in MDA-MB-231 was confirmed as incubation of cells with TNF-α resulted in a small and transient increase in JNK activity of approximately 4 fold at 15 minutes (4.02 ± 0.89 fold stimulation, $p < 0.01$) (Figure 4.1B). This JNK activation then returned almost to baseline at 60 minutes (1.53 ± 0.37 fold stimulation). In Figure 4.1C, TNF-α stimulated a concentration dependent activation of JNK signalling as measured at 30 minutes, with approximately 3.6 fold stimulation at 5 ng/ml. The expression of TNFR1 on the cell

surface of MDA-MB-231 has been previously confirmed by other studies (Hamaguchi et al., 2011). The subsequent experiments used TNF-α at 5 ng/ml for an incubation time of 30 minutes.

The inhibitory effect of ATP on TNF-α stimulated JNK activity was then tested using a pre-incubation time of 30 minutes. In Figure 4.2, TNF-α alone induced a small increase in JNK activity (3.26 ± 0.68 fold stimulation). Pre-incubation with ATP inhibited TNF-α mediated JNK signalling in a concentration-dependent manner, essentially abolishing the TNF-α response. An IC_{50} of approximately 0.7 µM was obtained over a number of experiments. This value was approximately 10 fold lower than that obtained in HUVECs as outlined in Chapter 3.

Similarly, pre-treatment of MDA-MB-231 with adenosine also inhibited TNF-α induced JNK activity in a concentration-dependent manner. Adenosine at 3 µM reduced the TNF-α induced JNK signalling by approximately half (% TNF-α stimulation = 50.23 ± 11.80 %, $p < 0.05$) (Figure 4.3). Based on several experiments, an IC_{50} of 2.85 ± 0.78 µM was obtained.

A

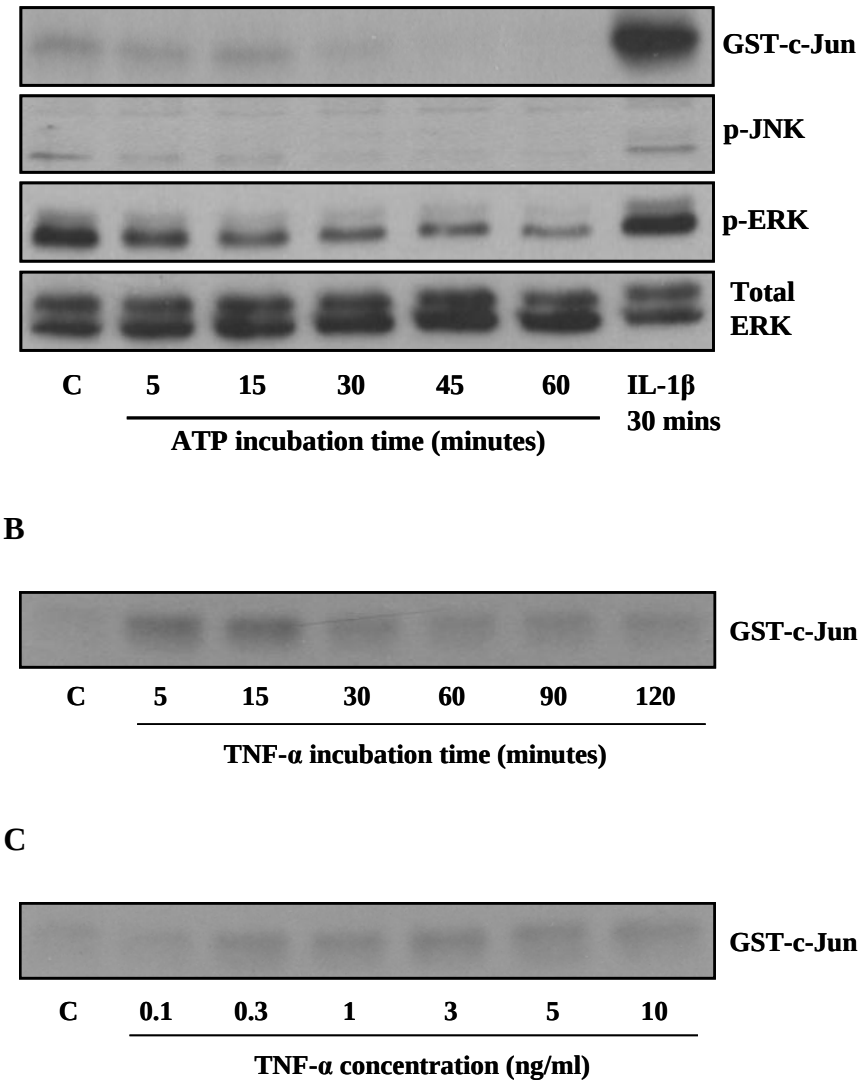
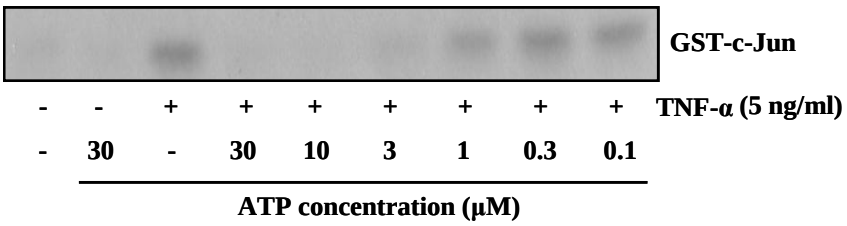


Figure 4.1: Time and concentration dependent MAPK signalling by ATP and TNF-α in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with ATP (100 µM) or TNF-α (10 ng/ml) for the times indicated above for (A) and (B) or at increasing concentrations of TNF-α (0.1 – 10 ng/ml) for 30 minutes for (C). JNK activity and ERK phosphorylation were assessed by *in vitro* kinase assay and Western blotting as described in section 2.5 and 2.6 respectively. n = 3 for A and B; n = 2 for C.

A



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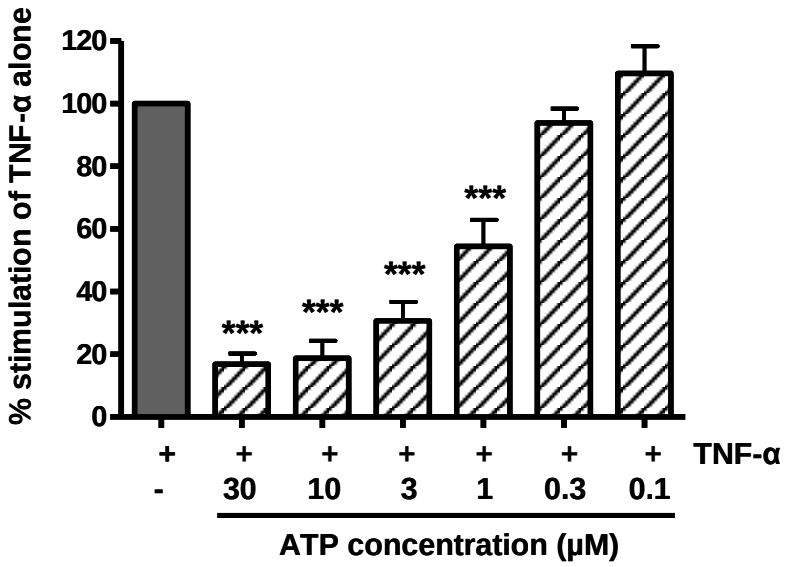


Figure 4.2: Concentration-dependent ATP mediated inhibition of TNF-α induced JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of ATP (0.1 – 30 μM) for 30 minutes, followed by TNF-α stimulation (5 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to TNF-α stimulation only.

A

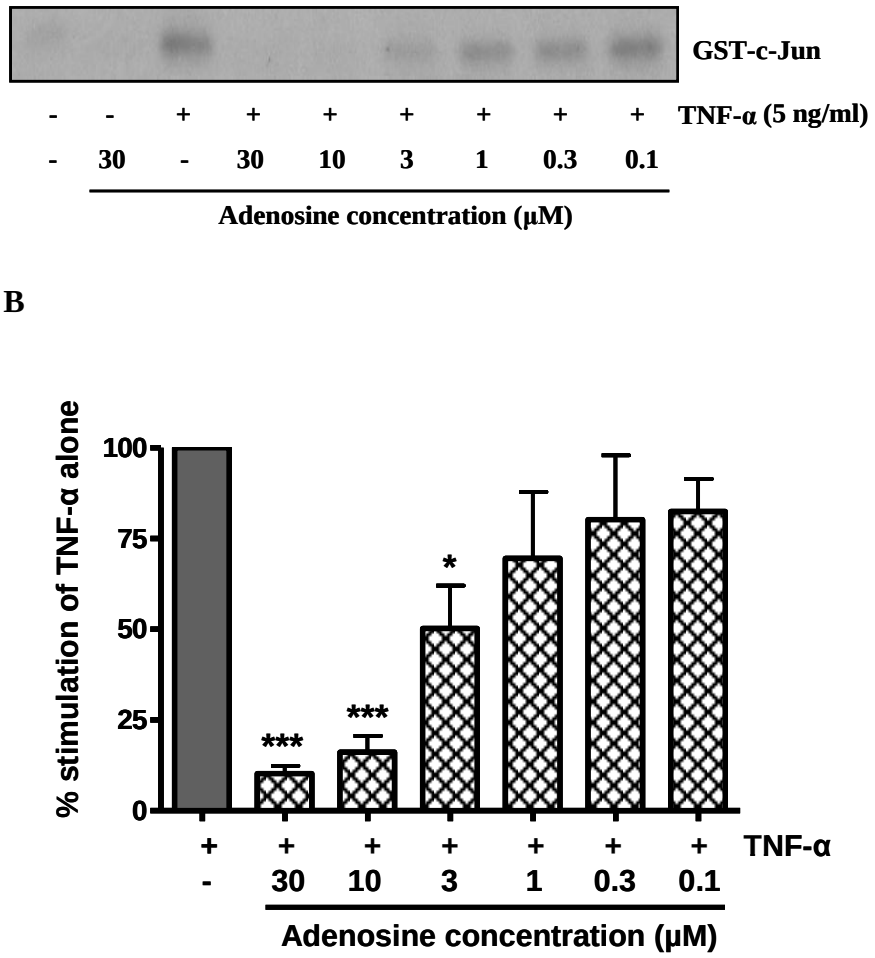


Figure 4.3: Concentration-dependent adenosine mediated inhibition of TNF-α stimulated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of adenosine (0.1 – 30 μM) for 30 minutes, followed by TNF-α stimulation (5 ng/ml) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, * $p < 0.05$ compared to TNF-α stimulation only.

4.2.2 ATP and adenosine mediated inhibition of IL-1β induced JNK signalling in MDA-MB-231

Due to the relatively low JNK signal stimulated by TNF-α, the effect of IL-1β was also examined in MDA-MB-231. The expression of the IL-1R1 was confirmed as incubation of MDA-MB-231 with IL-1β (10 ng/ml) stimulated a time and concentration dependent increase in JNK activity as shown in Figure 4.4. Maximum JNK activity was detected as early as 15 minutes (6.37 ± 0.38 fold stimulation, $p < 0.001$), which then returned towards basal level by 60 minutes (1.35 ± 0.57 fold stimulation). IL-1β also increased JNK signalling in a concentration-dependent manner. IL-1β at 5 ng/ml was sufficient to induce a JNK signal of approximately 6.2 fold stimulation. Thus IL-1β at 5 ng/ml for 30 minutes was used for the following experiments, in combination with ATP or adenosine. This concentration correlated with another study that also used 5 ng/ml of IL-1β to induce strong p38 MAPK phosphorylation in MDA-MB-231 (Park and Kwon, 2011).

Next, MDA-MB-231 cells were pre-incubated with increasing concentrations of ATP (0.1 to 30 μM) for 30 minutes, followed by IL-1β for a further 30 minutes as shown in Figure 4.5 Similar to the effect on TNF-α, ATP significantly inhibited IL-1β dependent JNK signalling over the low micromolar concentration range. ATP at 30 μM inhibited IL-1β mediated JNK activity to approximately 20% (% IL-1β stimulation: ATP + IL-1β = 13.28 ± 3.61 %, $p < 0.001$), with an IC_{50} value of 1.33 ± 0.26 μM was obtained over several experiments. This value compared well to that measured in HUVECs (2.34 ± 3.87 μM). In Figure 4.5, adenosine was also found to reduce IL-1β induced JNK signalling with similar efficacy and potency to that observed for ATP (% IL-1β stimulation: Adenosine 30 μM + IL-1β = 10.91 ± 3.49 %, $p < 0.001$), with an IC_{50} of approximately 1.8 μM. This value was however, approximately 10 fold lower than that observed in HUVECs.

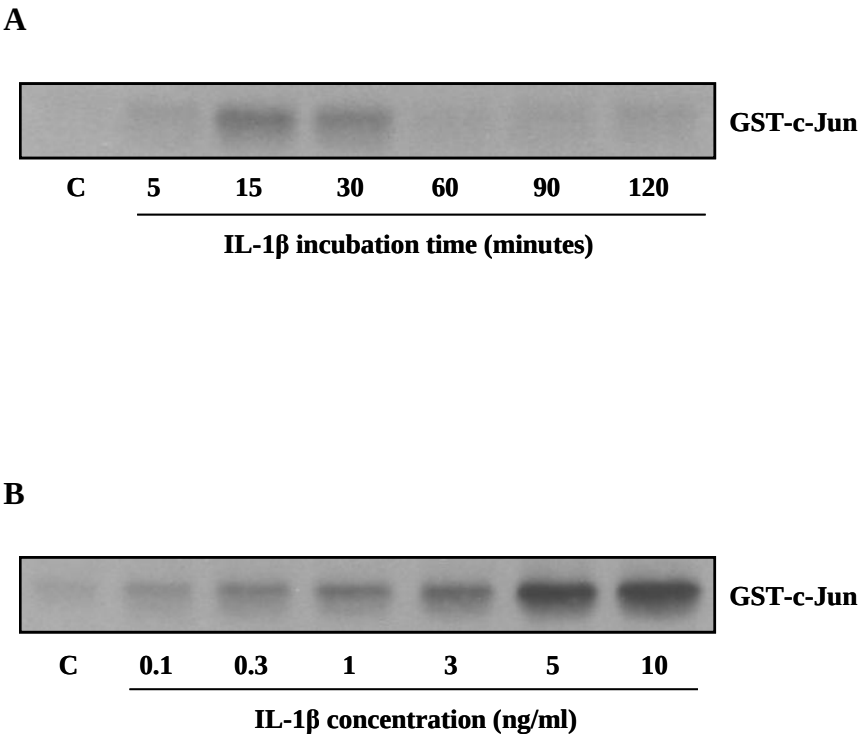


Figure 4.4: Time and concentration dependent JNK signalling by IL-1β in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with IL-1β (10 ng/ml) for the times indicated above for (A) or at increasing concentration of IL-1β (0.1 – 10 ng/ml) for 30 minutes for (B). JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. n = 3 for A; n = 2 for B.

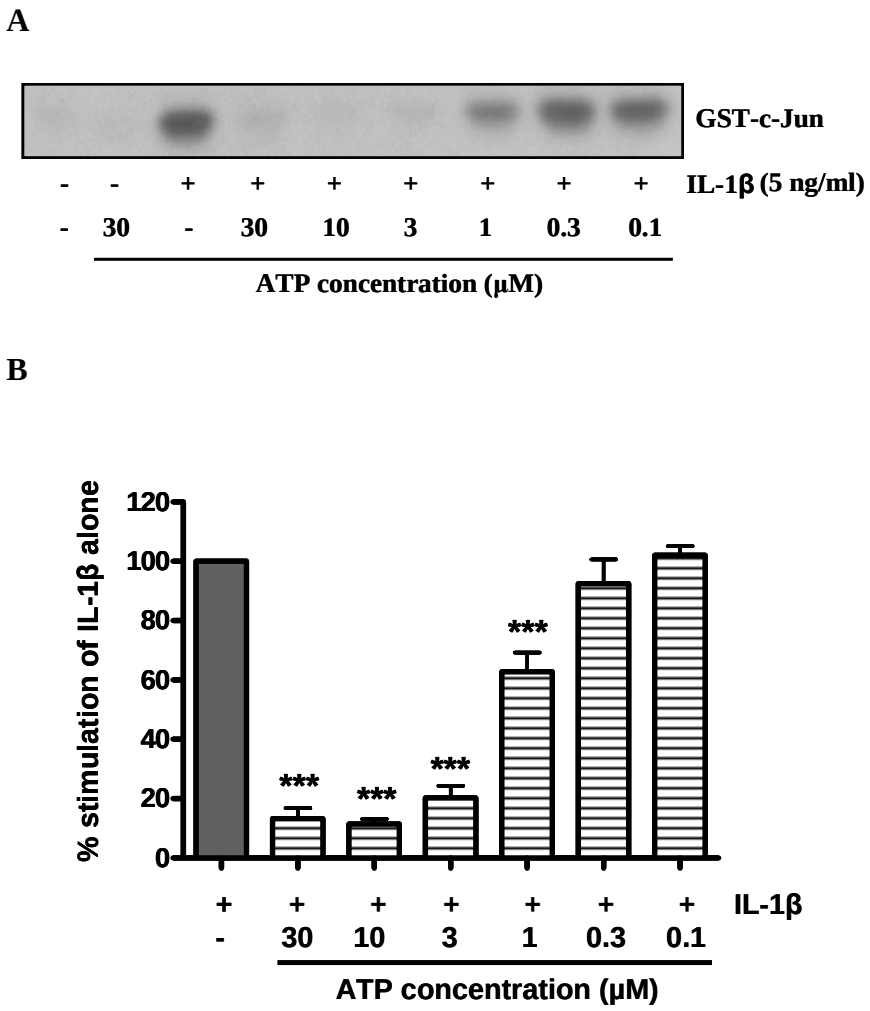


Figure 4.5: Concentration-dependent ATP inhibition of IL-1β mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of ATP (0.1 – 30 μM) for 30 minutes, followed by IL-1β stimulation (5 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to IL-1β stimulation only.

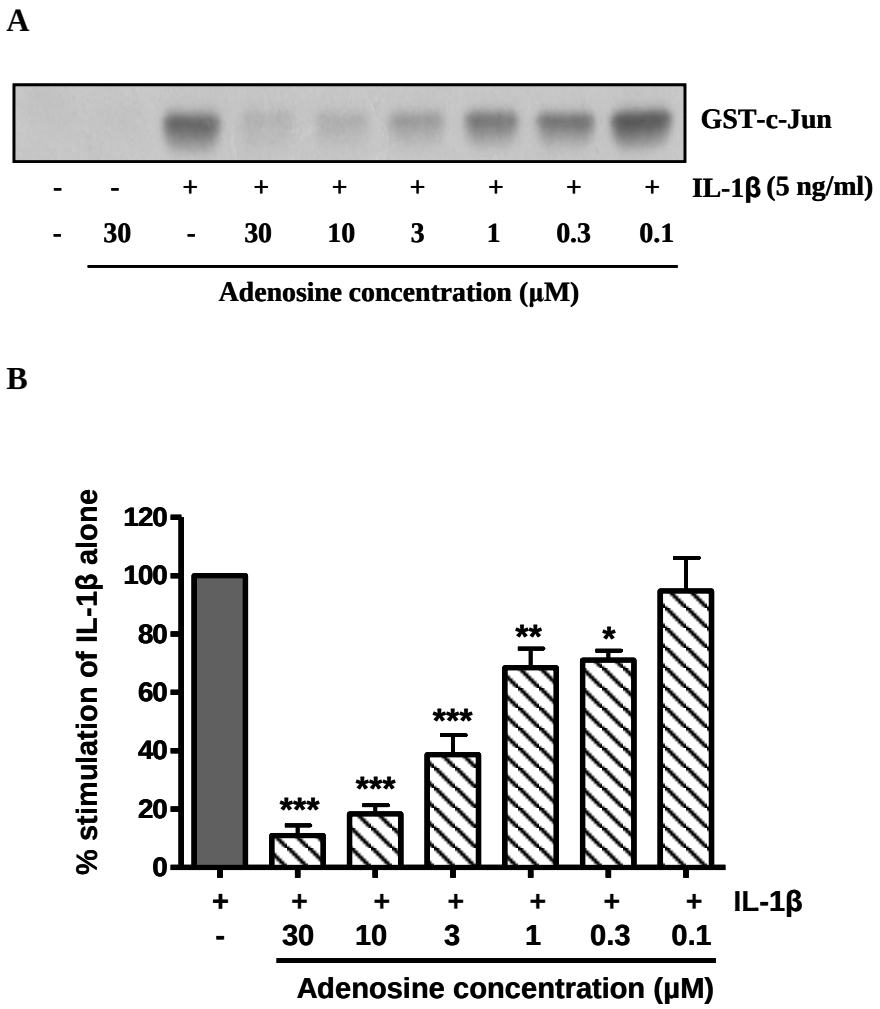


Figure 4.6: Concentration-dependent adenosine inhibition of IL-1β mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of adenosine (0.1 – 30 μM) for 30 minutes, followed by IL-1β stimulation (5 ng/ml) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to IL-1β stimulation only.

4.3 ATP mediated inhibition of UVC induced JNK signalling in MDA-MB-231

4.3.1 UVC activation of JNK signalling in MDA-MB-231

In order to investigate the specificity of the inhibitory effect of ATP and to relate inhibition to cellular outcomes relevant to cancer, UVC irradiation was used. Firstly, the kinetics of UVC induced JNK signalling at 30 J/m^2 was measured over 24 hours using *in vitro* kinase assay (Figure 4.7). As found in other studies (Chen et al., 1996b, Hamdi et al., 2005, Liu et al., 1995), sustained JNK activation was observed over the 24 hour duration, with an early maximum signal obtained 30 minutes following exposure to UVC (14.87 ± 2.43 fold stimulation, $p < 0.001$). Surprisingly, JNK activation was biphasic, levels decreased to near basal from 1 - 2 hours (2 hours: 0.82 ± 0.12 fold stimulation). At 4 hours, JNK activity gradually increased reaching a second peak of approximately 17 fold stimulation at 8 hours (17.29 ± 1.55 fold stimulation, $p < 0.001$). A high level of JNK activity was even detected at 24 hours following UVC exposure (12.07 ± 2.34 fold stimulation).

The concentration dependent activation of JNK signalling by UVC was then measured at 30 minutes (Figure 4.8). Even at 10 J/m^2, UVC stimulated a strong JNK signal (9.33 ± 2.73 fold stimulation), which increased further at both 30 and 60 J/m^2. Over a number of experiments, the EC_{50} calculated for UVC was approximately 15.7 J/m^2. Thus, for subsequent studies, a dose of 30 J/m^2 was used.

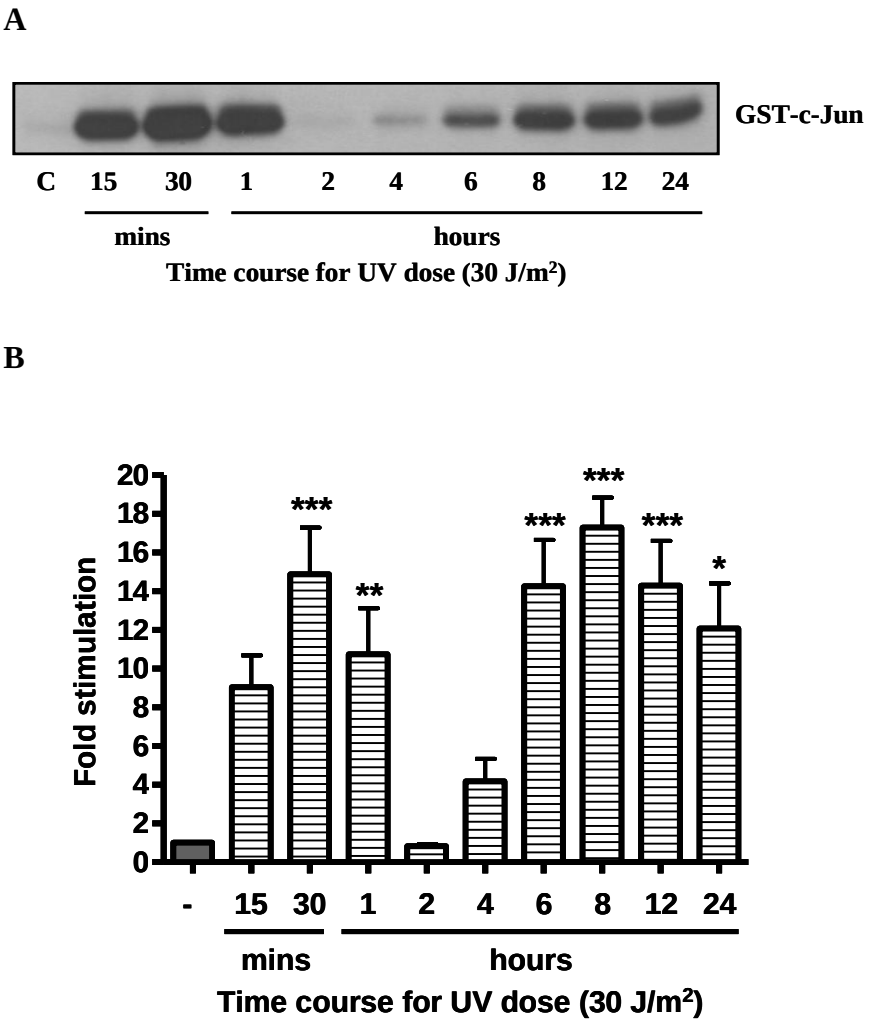


Figure 4.7: Time dependent JNK activation by UVC in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were exposed to UVC (30 J/m^2) for the times indicated above. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to unstimulated control.

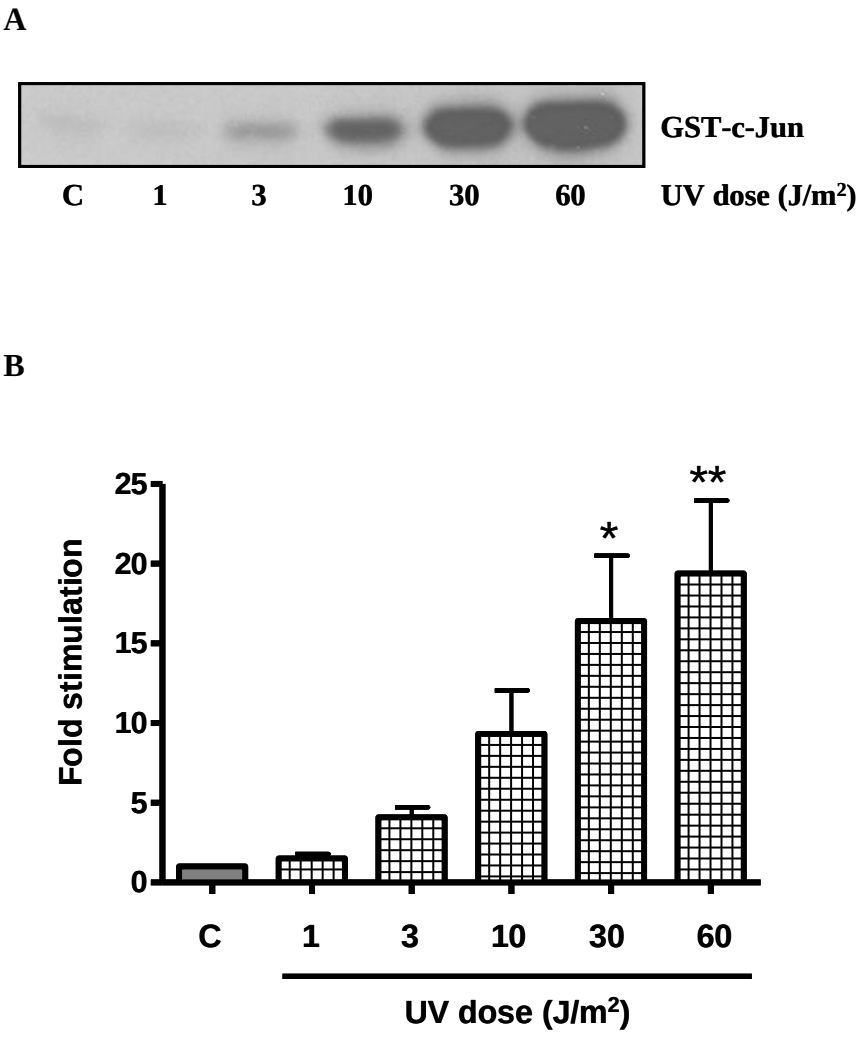


Figure 4.8: Concentration-dependent JNK activation by UVC in MDA-MB-231 cells

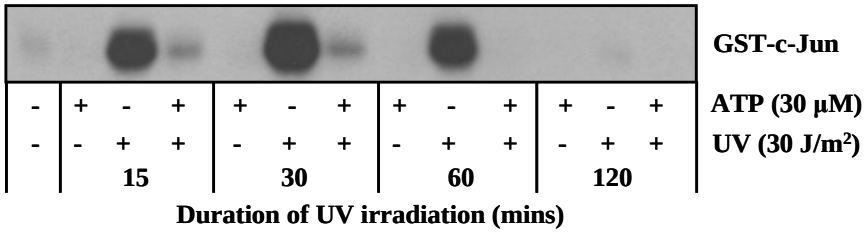
Cells, rendered quiescent for 24 hours, were exposed to increasing doses of UVC (1 - 60 J/m^2) for 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$, * $p < 0.05$ compared to unstimulated control.

4.3.2 Pre-incubation of ATP inhibited the early phase of UVC-mediated JNK signalling

The following experiments then attempted to characterise the inhibitory effect of ATP on UVC induced JNK activity. Cells were pre-incubated with ATP (30 µM) for 30 minutes, followed by exposure to UVC for different time periods (15 minutes – 2 hours) as shown in Figure 4.9. Over this time course, ATP alone did not stimulate an increase in the JNK signal. In contrast, ATP effectively inhibited UVC mediated JNK signalling at 15, 30 and 60 minutes by approximately 80 %. (Fold stimulation: 30 minutes: UVC = 13.89 ± 3.68; ATP + UVC = 2.93 ± 0.68, $p < 0.001$).

Therefore, based on pre-incubation with ATP at 30 minutes followed by UVC for another 30 minutes, the concentration dependent inhibition of JNK was studied as shown in Figure 4.10. Pre-incubation using ATP also reduced UVC dependent JNK signalling in a concentration dependent manner, with inhibition of approximately 70 % using 10 and 30 µM (% UVC stimulation: ATP 10 µM + UVC = 33.29 ± 8.46 %; ATP 30 µM ± UVC = 27.63 ±7.02; both $p < 0.001$). The IC_{50} for this ATP inhibitory effect was 6.87 ± 1.59 µM, which was slightly higher than those values obtained for TNF-α and IL-1β.

A



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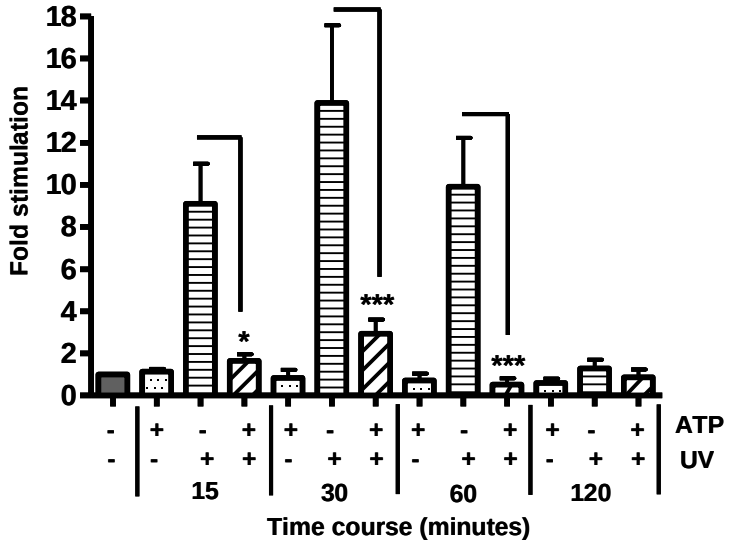

Figure 4.9: Pre-incubation with ATP inhibited the early phase of UVC mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with ATP (30 µM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for the times indicated above. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$, * $p < 0.05$ compared to UV only stimulation for the corresponding time points. The statistical difference between unstimulated control and UVC were not indicated in the graph.

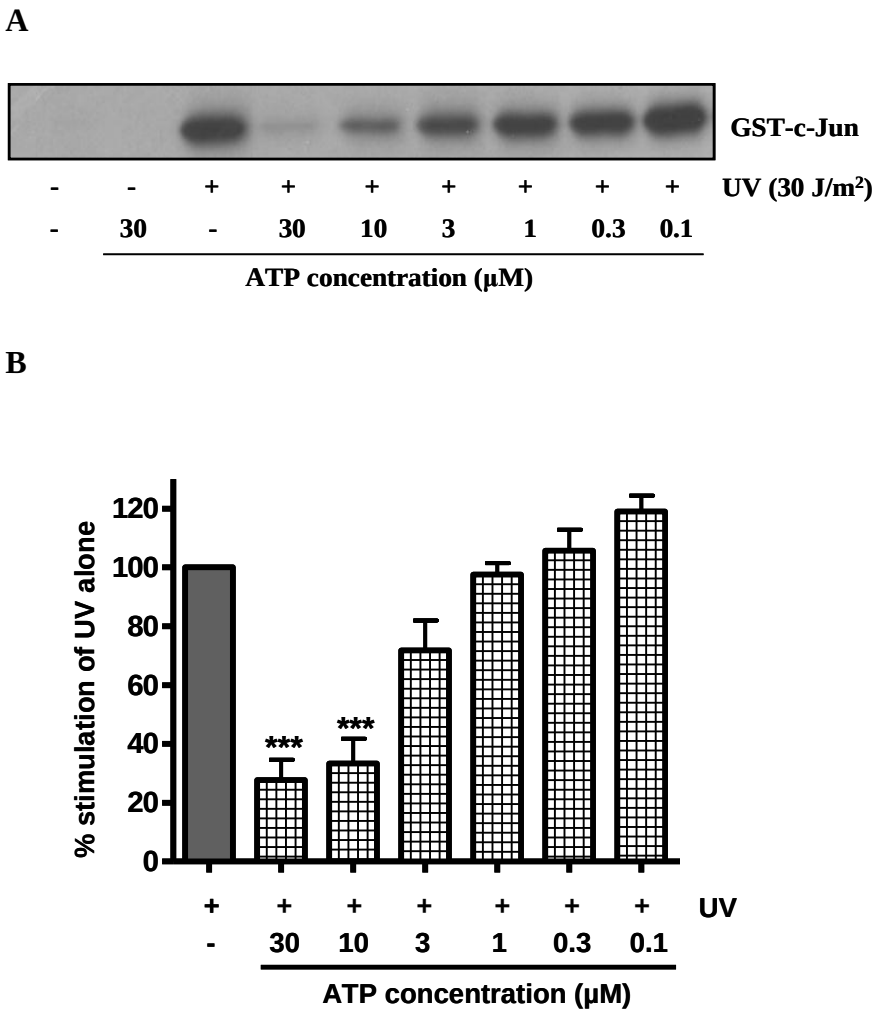

Figure 4.10: Concentration dependent ATP inhibition of UVC mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of ATP (0.1 – 30 µM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to UVC only stimulation.

4.3.3 Pre-incubation with ATP to examine the late phase of UVC mediated JNK signalling

In this section, the ability of ATP to inhibit the late phase of JNK signalling, at post 2 hours of exposure to UVC, was examined. Again, the pre-incubation of ATP for 30 minutes was applied, followed by UVC irradiation and incubation of cells for 4 to 24 hours. In figure 4.11, ATP was shown to reduce the UVC dependent JNK activity slightly at 6 hours onwards, but the inhibitory effect did not reach statistical significance. For instance, at the second peak of JNK signalling (8 hours), ATP produced an approximately 20 % inhibition (Fold stimulation: UVC = 15.55 ± 0.45; ATP + UVC = 12.14 ± 0.57).

In order to confirm the inhibitory effect of ATP in the late phase of JNK activation, ATP at increasing concentration of 3, 10 and 30 µM were tested at 30 minutes, 8 hours and 24 hours following exposure to UVC (Figure 4.12). As observed in figure 4.8, ATP at these concentrations significantly inhibited the JNK signal at 30 minutes. For example, ATP at 30 µM reduced the JNK signalling to baseline (Fold stimulation: UVC = 9.06 ± 2.26; ATP + UVC = 1.16 ± 0.35; $p < 0.001$). Pre-incubation of ATP at 30 µM slightly reduced the JNK activity at 8 hours as observed in the earlier figure, however at 24 hours, ATP significantly inhibited UVC induced JNK activation by approximately 65 % (Fold stimulation at 24 hours: UVC = 9.08 ± 2.06; ATP + UVC = 3.26 ± 0.81, $p < 0.05$). Overall, these data implied that ATP could also alter the late JNK signal induced by UVC, however the effect was time dependent.

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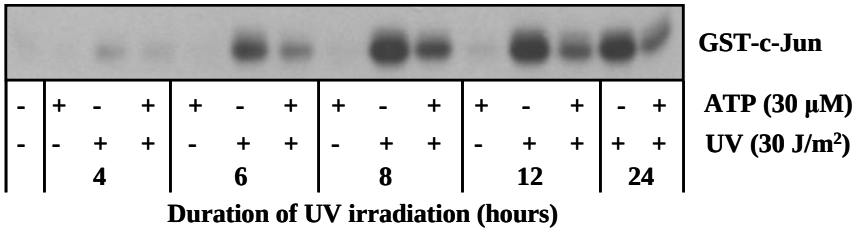

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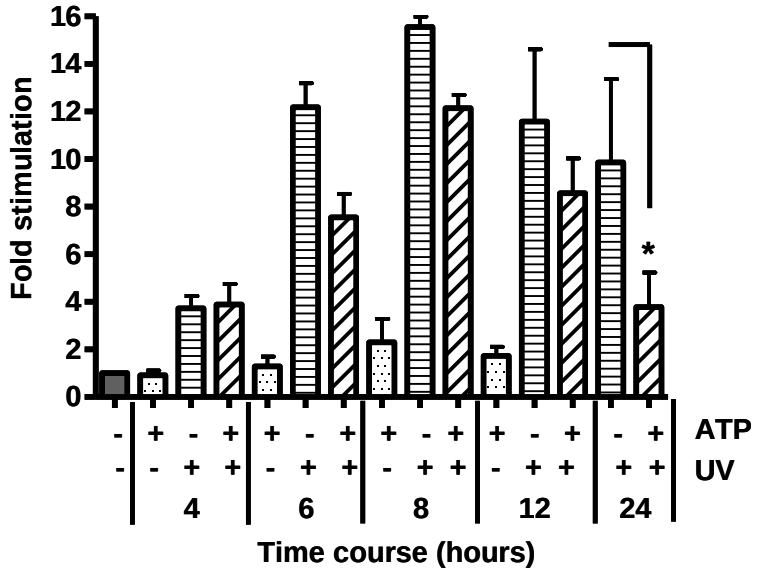

Figure 4.11: Pre-incubation of ATP slightly inhibited the late phase of UVC mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with ATP (30 µM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for the times indicated above. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test. * $p < 0.05$ compared to UVC only at the same time point. The statistical differences between unstimulated control and UVC or ATP + UVC were not indicated in the graph.

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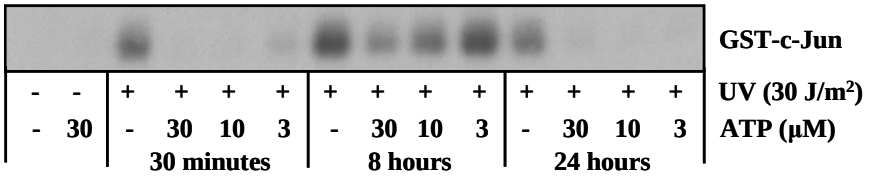

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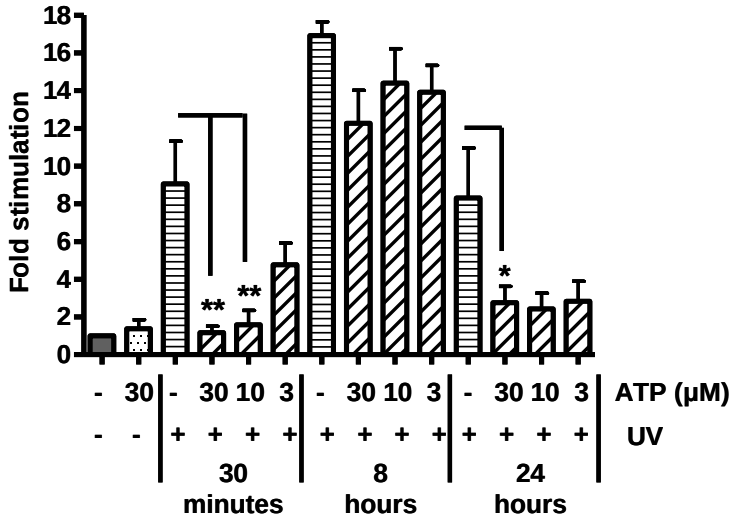

Figure 4.12: Pre-incubation with ATP inhibited UVC mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of ATP (3 – 30 μM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for the indicated times (30 minutes, 8 hours and 24 hours). JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. ** $p < 0.01$ compared to UV only stimulation for the corresponding time points. The statistical differences between unstimulated control and UVC or ATP + UVC were not indicated in the graph.

4.3.4 Post-incubation with ATP did not inhibit the UVC mediated JNK signalling

Since pre-incubation with ATP minimally affected the late phase of UVC dependent JNK activity, ATP was applied prior to the second increment of JNK activity. Thus at 6 hours post UVC irradiation, 30 μM of ATP was added into the supernatant and samples were assayed at the time points as indicated in Figure 4.13. At all time points measured, ATP did not alter the JNK signalling mediated by UVC. For instance, at 12 hours post UVC irradiation, UVC alone induced an approximate 10 fold (10.09 ± 1.46 fold stimulation) increase in JNK activity whilst combined ATP and UVC treatment produced approximately 9.6 fold (9.55 ± 1.88 fold stimulation). Therefore, the marginal inhibitory effect of ATP in the late phase of JNK signalling may be a delayed inhibition manifested from the early phase.

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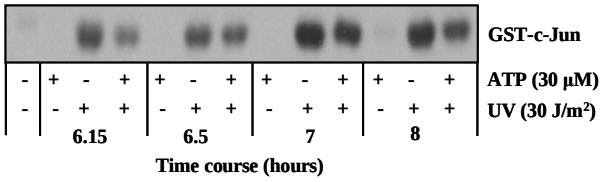

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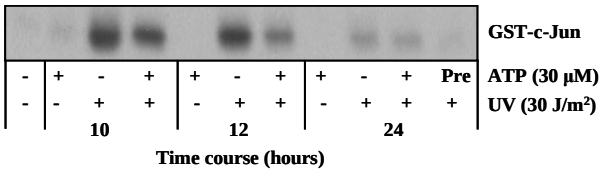

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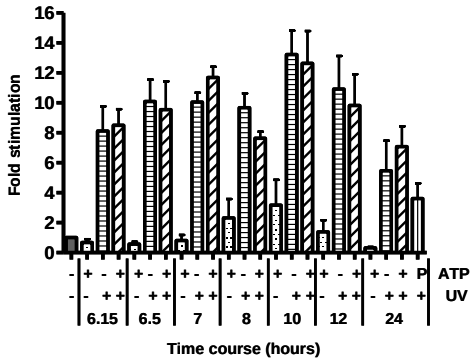

Figure 4.13: Post 6 hours addition of ATP did not affect UVC mediated JNK signalling (throughout 24 hours) in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were exposed to UVC (30 J/m^2) for the indicated time points, with or without the addition of ATP (30 µM) at 6 hours post-UVC exposure. P indicated pre-incubation of ATP for 30 minutes, followed by UVC irradiation. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) and (B) showed a representative blot and (C) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no differences were found between UVC and ATP + UVC at the same corresponding time points. The statistical differences between unstimulated control and UVC or ATP + UVC were not indicated in the graph.

4.4 Characterisation of the purinergic receptors involved in the inhibitory effects of ATP on JNK signalling in MDA-MB-231: The role of $P2Y_2$ receptor

Next, the P2Y receptor responsible for the inhibitory effect in MDA-MB-231 cells was investigated as shown in Figure 4.14. A recent study has indicated that $P2Y_2$ is expressed on the cell surface of MDA-MB-231 (Li et al., 2011). Due to the lack of potent and selective $P2Y_2$ antagonists to investigate the role of $P2Y_2$ receptor (Jacobson, 2010), UTP, that activates $P2Y_2$ receptor equipotently as ATP, was used. In figure 4.12, pre-incubation with UTP did not inhibit UVC mediated phosphorylation of either JNK or p38 MAPK, demonstrating that $P2Y_2$ receptor is not responsible for the inhibitory effect of ATP in MDA-MB-231 cells.

4.5 The role of $G_{q/11}$ in the inhibitory effect of ATP in UVC mediated JNK signalling in MDA-MB-231

As described in the previous results chapter, pharmacological inhibition of $G_{q/11}$ by YM254890 partially reversed the inhibitory effect of ATP in IL-1β mediated JNK signalling in HUVECs. Thus the role of $G_{q/11}$ was then clarified by pre-treatment of cells with YM254890 (Figure 4.15). Consistent with the previous results, ATP at 30 μM inhibited UVC induced JNK activity by approximately 75 % (% UVC stimulation = 26.13 ± 5.56 %). Pre-treatment with YM254890 at 100 nM was sufficient to completely reverse the inhibitory effect of ATP (% IL-1β stimulation: 97.30 ± 2.28 %). Thus, a $G_{q/11}$ coupled P2Y receptor is responsible for the inhibitory effect of ATP in MDA-MB-231.

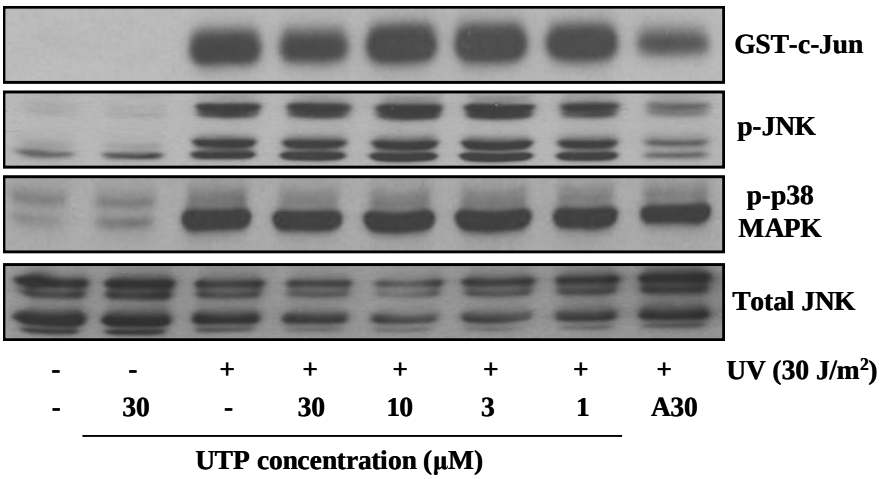

Figure 4.14: Incubation of UTP did not alter the UVC mediated JNK and p38 MAPK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of UTP (0.1 – 30 µM) or ATP 30 µM (A30) for 30 minutes, followed by UVC stimulation (30 J/m^2) for another 30 minutes. JNK activity and phosphorylation of p38 MAPK were assessed by *in vitro* kinase assay and Western blotting as described in sections 2.5 and 2.6 respectively, n = 2.

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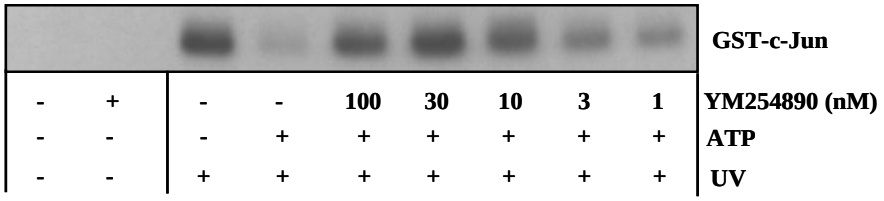

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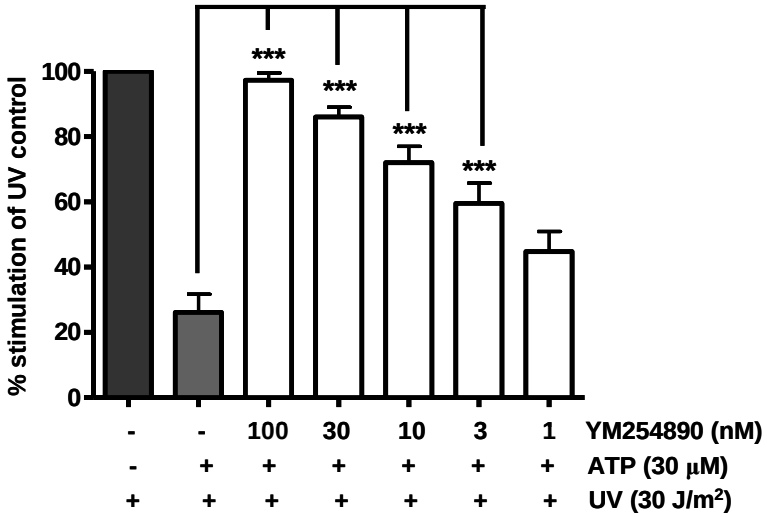

Figure 4.15: $G_{q/11}$ mediated the inhibitory effect of ATP in UVC mediated JNK signalling in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentration of YM254890 (1 – 100 nM) for 15 minutes, followed by ATP (30 µM) for 30 minutes and UVC (30 J/m^2) for a further 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 5. Statistical analysis was performed using one way ANOVA with Dunnett's post test. *** $p < 0.001$ compared to combined ATP and UV stimulation. The statistical difference between UVC only and ATP + UVC was not indicated in the graph.

4.6 ATP mediated inhibition of UVC induced JNK signalling in SW1353

To test the cell specificity of the inhibitory effect of ATP, another cancer cell line, namely the chondrosarcoma line SW1353 was also used. Preliminary experiment demonstrated a small increase in ERK phosphorylation at 5 minutes with ATP incubation, but ATP did not induce any changes in JNK activity over 60 minutes (results not shown). Therefore, P2Y receptors are indeed present on the cell surface membrane of SW1353.

4.6.1 ATP inhibited the UVC mediated JNK signalling in SW1353

The ability of ATP to inhibit the early phase of UVC induced JNK signalling, was then examined by pre-treating the cells with ATP at 0.1 to 30 μM for 30 minutes, followed by 30 minutes of UVC irradiation (Figure 4.16). A maximal inhibition of approximately 40 % was detected using ATP at a concentration of 30 μM (% UVC stimulation: ATP 30 μM + UVC = 58.46 ± 7.90 %, $p < 0.05$), with an IC_{50} of approximately 37 μM. Thus, the inhibitory effect of ATP in UVC induced JNK signalling was not a cell type specific phenomenon, but relied on the expression of the P2Y receptor on the cancer cell surface.

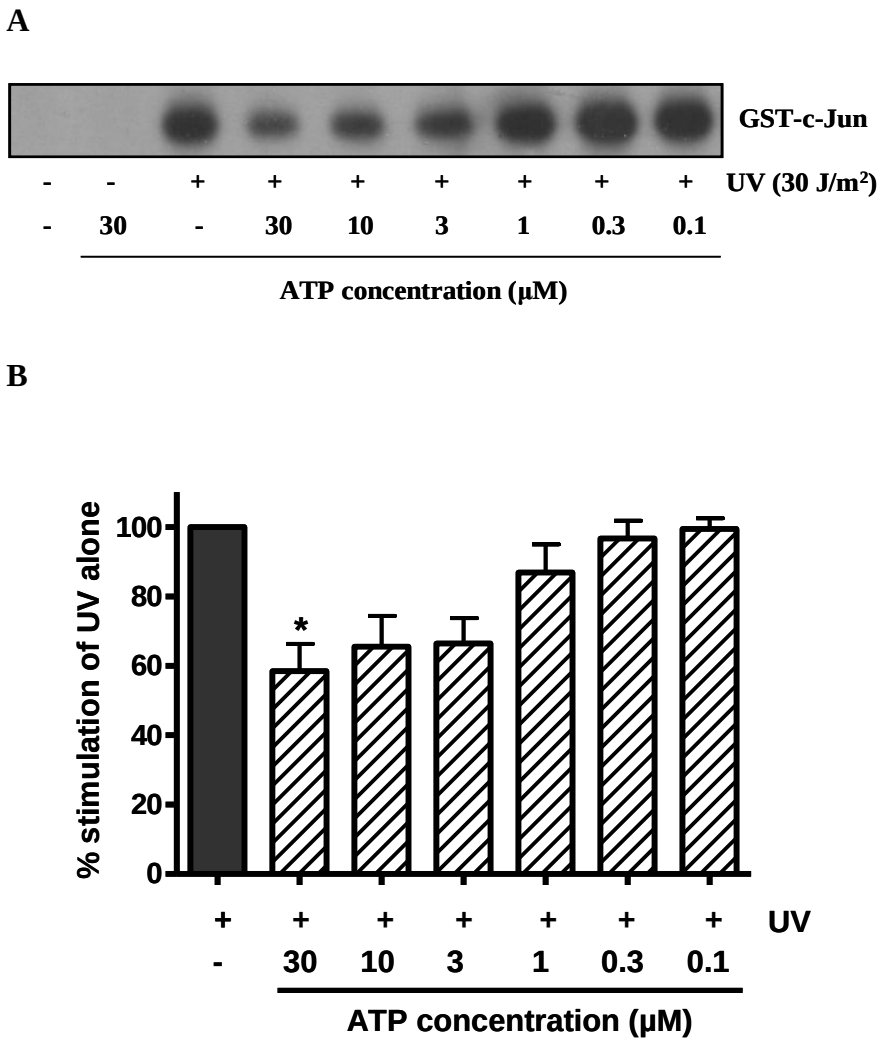

Figure 4.16: Concentration dependent ATP inhibition of UVC mediated JNK signalling in SW1353 cells

Cells, rendered quiescent for 24 hours, were incubated with increasing concentrations of ATP (0.1 – 30 μM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for another 30 minutes. JNK activity was assessed by *in vitro* kinase assay as described in section 2.5. (A) showed a representative blot and (B) showed the densitometric data for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test. * $p < 0.05$ compared to UVC only stimulation.

4.7 The effect of ATP and JNK inhibitor in UVC mediated apoptosis in MDA-MB-231

Since sustained JNK signalling and UVC were shown to cause apoptosis in various cell lines (Chen et al., 1996a, Dunkern et al., 2001, Hamdi et al., 2005, Liu et al., 1995, Wu et al., 2002), the apoptotic effect of UV, in combination with ATP, was then measured. Cells were stained with a phospholipid phosphatidylserine dye, phycoerythrin (PE) annexin V, and nucleic acid dye, 7-amino-actinomycin (7-AAD), to reveal the cell population that are healthy, early apoptosis, late apoptosis and necrotic in FACS analysis. The % apoptotic cells mentioned in the following sections were a combination of cell population of early apoptosis, late apoptosis and necrotic.

4.7.1 The role of JNK in the UVC mediated apoptosis in MDA-MB-231

In order to understand the role of JNK in the apoptosis effect of UVC in this breast cancer cell line, the pharmacological JNK inhibitor, SP600125, was applied for 30 minutes prior to exposure to UVC irradiation. The cell population was then analysed after 24 hours as shown in Figure 4.17. In the untreated sample, the majority of the cells were healthy (% healthy cells = 90.53 ± 0.91 %). However, the application of the JNK inhibitor slightly increased the % of apoptotic cells, but the results were not statistically significant. For example, SP600125 at 10 μM produced approximately 31 % (31.03 ± 7.74 %) of apoptotic cells, suggesting that JNK plays a negative role in the basal apoptotic rate of MDA-MB-231. At 24 hours, exposure to UVC produced significant cell death (% apoptotic cells = 38.03 ± 3.75 %, $p < 0.05$ compared to unstimulated control). However, inhibition of JNK did not produce a statistical significant increase in the apoptotic effect of UVC (SP600125 10 μM + UVC = 45.95 ± 7.77 % apoptotic cells). Thus JNK did not play an important role in mediating the apoptotic effect of UVC in this cell line.

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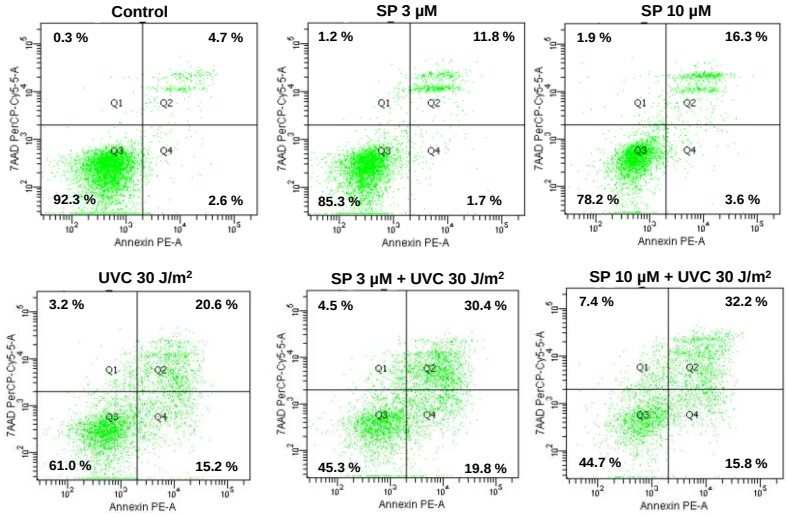

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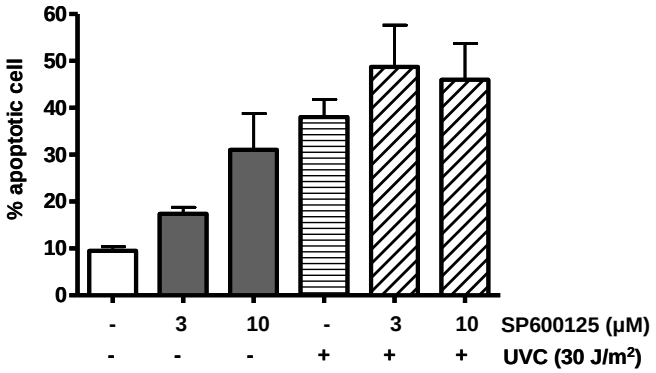

Figure 4.17: The effect of JNK inhibition on UVC mediated apoptosis in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with SP600125 (3 and 10 µM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for another 24 hours. The cell population was collected, stained and assessed by flow cytometry as described in section 2.6. (A) showed a representative data and (B) showed the % of apoptotic cells for several independent experiments, expressed as mean ± SEM, n = 3. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no differences were found between UVC only and SP600125 + UVC. The statistical differences between unstimulated control and SP600125, UVC or SP600125 + UVC were not indicated in the graph.

4.7.2 The role of ATP in UVC mediated apoptosis in MDA-MB-231

Next, the role of ATP was then studied by pre-incubation of ATP, followed by UVC irradiation as shown in Figure 4.18. Similar to the effect of the JNK inhibitor, ATP alone slightly increased the amount of apoptotic cells compared to untreated cells (% apoptotic cells: untreated = 17.04 ± 1.95 %; ATP 100μM = 27.93 ± 3.92 %). UVC irradiation consistently produced significant cell death (47.34 ± 6.35 % apoptotic cells, $p < 0.01$ compared to untreated control). Combining ATP and UVC, the apoptotic effect was unaltered (ATP 100 μM + UVC = 53.75 ± 7.70 %), confirming the results from the previous section that JNK minimally affected the apoptotic pathway mediated by UVC in MDA-MB-231 cells, and that ATP plays no role in this process.

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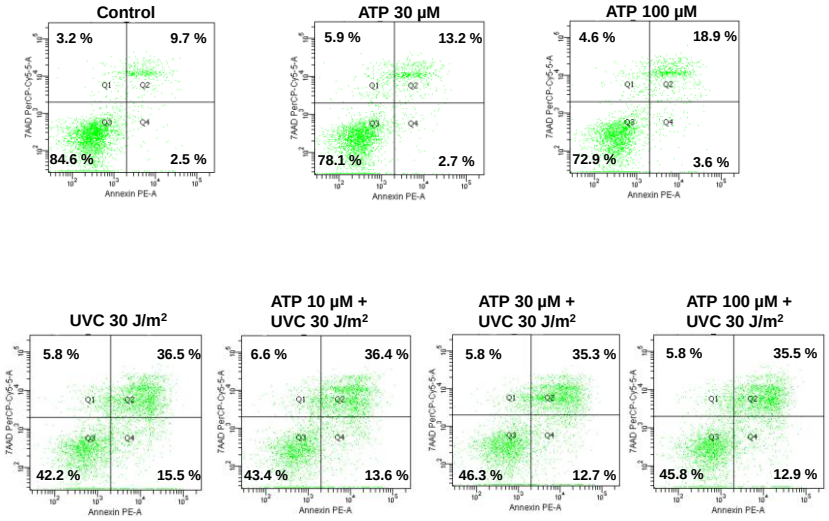

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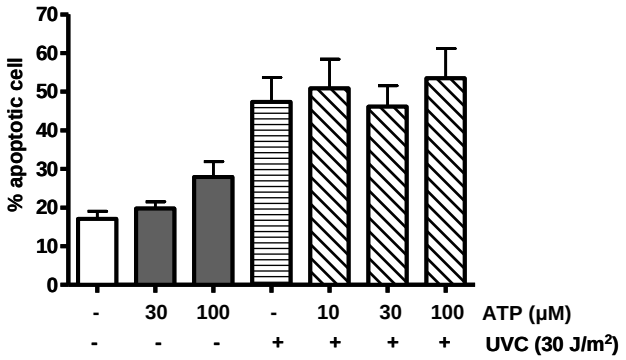

Figure 4.18: The effect of ATP in UVC mediated apoptosis in MDA-MB-231 cells

Cells, rendered quiescent for 24 hours, were incubated with ATP (10, 30 and 100 µM) for 30 minutes, followed by UVC stimulation (30 J/m^2) for another 24 hours. The cell population was collected, stained and assessed by flow cytometry as described in section 2.6. (A) showed a representative data and (B) showed the % of apoptotic cells for several independent experiments, expressed as mean ± SEM, n = 4. Statistical analysis was performed using one way ANOVA with Dunnett's post test, but no differences were found between UVC only and different concentrations of ATP + UVC. The statistical differences between unstimulated control and ATP, UVC or ATP + UVC were not indicated in the graph.

4.8 Discussion

In this chapter, the inhibitory effect of P2Y receptors upon cytokine-mediated JNK signalling was determined using the breast cancer cell line MDA-MB-231 and the chrondrosarcoma line SW1353. These results confirmed the hypothesis that the inhibitory effect of GPCR activation on JNK signalling may be a widespread phenomenon. Furthermore, this can be extended to other activating agents, for example UVC (see below).

Similar to HUVECs, pre-incubation with ATP and adenosine in these cancer cell lines inhibited JNK activity induced by both TNF-α and IL-1β in a concentration dependent manner. It was found that the IC_{50} of ATP and adenosine observed in MDA-MB-231 were lower than those observed in HUVECs, suggesting that this effect may be more relevant to the pathophysiology associated with cancer cell lines, rather than with primary cultured cells. The lower IC_{50} value could be due to an increase in basal ATP release already present in the serum-starved media which would allow a threshold level of inhibition to be obtained with lower concentrations of exogeneous ATP. Metastatic breast cancer cells can leak or release large quantities of ATP into its surroundings (approximately 100 nM after serum-starvation overnight) (Kawai et al., 2008), possibly resulting in a higher basal concentration of ATP compared to unstimulated HUVECs (approximately 50 nM or 40 pM concentration depending on the design of study) (Arakaki et al., 2003, Moser et al., 2001).

A second possibility is the overexpression of purinoceptors in cancer cells, as suggested for P2Y and A_{2B} receptors (Bianco et al., 2005). This has not been extensively studied nor have levels been quantified in cancer cell lines. However, an increase in $P2Y_2$ and $P2Y_4$ in colon cancer compared to neighbouring tumour-free tissues is reported (Nylund, 2004), whilst $P2X_7$ expression is linked to metabolic cancerous changes in breast lobules (Slater et al., 2004). Based on a combination of

these effects, cancer cells conferred a higher sensitivity towards the inhibitory effect of ATP and adenosine.

The finding that ATP inhibited UVC mediated JNK activity was unpredicted, as ATP did not affect the sorbitol and anisomycin induced JNK signalling in HUVECs as found in Chapter 3. In contrast to the short term JNK activity induced by cytokines, UVC produced a biphasic JNK signal that could be observed until 24 hours. Similar prolonged JNK activity by UVC exposure was also observed in other cell lines, such as Hela, human fibroblasts, HEK293T and Jurkat T cells (Chen et al., 1996b, Hamdi et al., 2005, Liu et al., 1995). However the biphasic nature of JNK signalling has been only shown in one study using Hela cells exposed to 40 J/m^2 of UVC, with a similar JNK activity reduction at the 2 hour time point (Liu et al., 1995). Due to the differences in the time points measured, the biphasic nature of the UVC was not shown in other studies.

Further characterisation of the inhibitory properties of ATP in UVC treated cells revealed that pre-incubation of ATP inhibited both the short and long term JNK signalling induced by UVC. However the inhibitory effect on long term JNK signalling (6 – 24 hours) was not significant despite the addition of ATP at 6 hours post-UV irradiation. Several studies have suggested that the different durations of JNK activation by UVC are initiated by different mechanisms. UVC activates early phase JNK signalling via assemble and internalisation of surface membrane receptors, including TNFR1, CD95/Fas receptor and EGFR (Rosette and Karin, 1996). UVC was also shown to stimulate ligand-independent TNFR1 activation and recruitment of FADD to TNFR1, with subsequent activation of caspase 8 and 10 (Sheikh et al., 1998), although this study did not link TNFR1-FADD signalling to JNK. Another study has shown that FADD only marginally activates JNK signalling (Liu et al., 1996). As P2Y activation can inhibit TNF-α mediated JNK activity, ATP might also inhibit UVC induced JNK signalling by preventing the recruitment of signal adaptor proteins to TNFR1.

Another member of TNF family, namely Fas/CD95 receptor was also found to mediate UVC dependent JNK signalling and apoptosis via Daxx binding protein (Wu et al., 2002). Dominant negative Daxx inhibited UVC mediated JNK activation in HEK293, but dominant negative FADD did not (Wu et al., 2002), suggesting that only specific downstream signalling of Fas/CD95 is linked to JNK activation. However evidence to date has shown that PKC activation in Jurkat T cells inhibited the downstream signalling of Fas/CD95 by preventing the recruitment of FADD and pro-caspase 8 to the death-inducing signalling complex (DISC) formation (Gomez-Angelats and Cidlowski, 2001). This implies that GPCRs coupled to $G_{q/11}$ and PKC could negatively regulate Daxx recruitment to Fas/CD95 to inhibit JNK signalling.

Another non-cytokine related membrane receptor, EGFR, also mediates downstream signalling in response to UVC exposure through phosphorylation of EGFR at Tyr1068 (Sachsenmaier et al., 1994, Cao et al., 2008). The tyrosine kinase inhibitor tyrphostin was found to inhibit UVC activated c-Jun in Hela cells (Devary et al., 1992) whereas the EGFR inhibitors PD153035 and AG1478 mediated UVC induced ERK, p38 MAPK, JNK, NF-κB, and PI3K signalling in mouse dendritic and skin cells (Cao et al., 2008, El-Abaseri et al., 2005). Therefore, P2Y receptors could inhibit UVC mediated transactivation by an unknown mechanism. However, in contrast, $P2Y_1$ and $P2Y_2$ activation in various cell lines has been shown to induce Src binding to the receptor, which then transactivates EGFR to mediate ERK signalling and cell proliferation (Buvinic et al., 2007, Liu et al., 2004b, Luke and Hexum, 2008, Milenkovic et al., 2003). Hence, transactivation of EGFR by P2Y in MDA-MB-231 is more likely to increase the UVC mediated JNK activation, rather than reduce JNK activity as observed in this chapter. In addition, JNK signalling induced by EGFR is significantly lower than UVC (Bost et al., 1997), indicating that this pathway alone is not sufficient to explain the strong activation of JNK by UVC.

Correspondingly, the lack of effect of ATP on the second phase of JNK could also have a molecular basis. In addition to the activation of cell surface receptors, UVC

also initiates DNA damage to promote long term JNK activation as observed in melanoma cells and fibroblasts (Adler et al., 1995a). Deficiency in both transcription-coupled and nuclear excision repair also induces greater JNK signalling and apoptosis respectively in response to UVC (Dunkern et al., 2001, Hamdi et al., 2005), supporting a non-redundant role of DNA damage in JNK activation and apoptosis for UVC. The results in this chapter demonstrate that ATP minimally affects the long term JNK activity initiated by UVC, suggesting that ATP is ineffective against DNA damage as it functions to inhibit receptor driven JNK activity. This minimal late inhibition may be due to a 'spill-over' inhibitory effect from the early phase of JNK signalling. Nevertheless, it cannot be excluded that ATP might inhibit sustained JNK signalling via induction of MKP-1, as increased expression of MKP-1 is reported to inhibit long term JNK signalling by UVC (Hamdi et al., 2005, Liu et al., 1995). Collectively, these studies indicate that multiple pathways are involved in the activation of JNK activity by UVC, but only the receptor dependent-early phase is susceptible to ATP inhibition. The possible site of inhibition for ATP in UVC mediated JNK signalling is summarised in Figure 4.19.

Additional studies were carried out to characterise the P2Y receptor(s) involved in the inhibitory effect of ATP in UVC induced JNK signalling. Time constraints did not allow this to be completed fully, nevertheless results in this chapter exclude a role for $P2Y_2$. Pre-incubation with UTP did not inhibit UVC-mediated JNK signalling, suggesting that $P2Y_2$ expression in MDA-MB-231 may not be sufficiently high to mediate the inhibitory effect of ATP. This finding is surprising as the limited studies on P2Y expression in MDA-MB-231 conducted to date indicate the presence of $P2Y_2$ (Li et al., 2011). Hence, further RT-PCR experiment revealing the complete P2Y subtype profile could be performed in order to understand the pharmacological basis of the inhibitory effect of ATP in MDA-MB-231 cells. Of the remaining candidates $P2Y_1$, that couples only to $G_{q/11}$ (Alexander et al., 2011), is most likely since the application of the $G_{q/11}$ inhibitor YM254890 completely reverses the inhibitory effect of ATP in the early phase of UVC mediated JNK signalling.

However, this receptor subtype is more responsive to ADP (Alexander et al., 2011), thus pre-treatment of cells with ADP or $P2Y_1$ agonist could clarify this possibility.

Irradiation of cells with UVC has commonly produced apoptotic outcomes in a variety of cell lines, as observed in this study (Berglund et al., 2004, Dunkern et al., 2001, Godar et al., 1994, Wu et al., 2002). The initial assumption made was that persistent JNK signalling would mediate the apoptotic effect of UVC in MDA-MB-231 cells. However, this assumption was not bourne out in this study. Firstly, it was found that the JNK inhibitor SP600125 used as a positive control enhanced apoptosis, suggesting that basal JNK activity was involved in mediating cell survival. This observation was supported using other approaches, incubation with SP600125 in MDA-MB-231 caused G2/M arrest with a slight increase in the sub-G1 peak indicative of cell apoptosis (Mingo-Sion et al., 2004). Knockdown of the kinase upstream of JNK, MKK4, in MDA-MB-231 also showed increased apoptosis related to an increase in caspase 3 activity and PARP cleavage (Wang et al., 2004).

Furthermore, it was found that SP600125 had no effect on UVC mediated apoptosis in MDA-MB-231. The lack of effect of SP600125 was not attributed to its possible degradation over the 24 hour duration as SP600125 produced sustained inhibition of phosphorylation of c-Jun mediated by PMA over 48 hours (Mingo-Sion et al., 2004). A seminal study which examined the role of JNK in apoptosis demonstrated that MEFs derived from JNK knockout mice ($JNK1^{-/-}$ and $JNK2^{-/-}$) were protected from UVC induced apoptosis due to inhibition of cytochrome c release (Tournier et al., 2000). Another study demonstrated that dominant negative JNK and SP600125 treatment inhibited UVC mediated cytochrome c release, caspase 9 and caspase 3 activation in lung carcinoma (Lo et al., 2004). However other approaches showed that the inhibitory effect of SP600125 in UVC mediated apoptosis was only observed during the pre-incubation of 48 hours, but not for 30 minutes (Berglund et al., 2004, Lo et al., 2004). It was thus suggested that JNK signalling can lead to the apoptotic mitochondrial caspase pathway, but prolonged pre-treatment of SP600125 was

required to condition the cells for pronounced JNK inhibition. The experiments in this chapter used a pre-incubation time of 30 minutes, however longer pre-incubation times could have been utilised. In addition, sustained JNK inhibition could have been achieved by overexpression of the MAPK phosphatases, such as MKP-1 and MKP-2 (Al-Mutairi et al., 2010).

Thus, a similar hypothesis that sufficient inhibition of sustained JNK is required to inhibit UVC mediated apoptosis could be applied to explain the ineffectiveness of ATP in influencing the UVC dependent apoptosis. Results from this chapter could not elucidate the functional effect of JNK inhibition by ATP. One additional area of study is the effect of JNK in regulating cell cycle progression, proliferation and migration. Recent studies have indicated that different isoforms of JNK can participate differentially in regulating proliferation, as $JNK1^{-/-}$ demonstrated slower proliferation rates whereas $JNK2^{-/-}$ exhibited increased proliferation (She et al., 2002). However, inhibition of JNK2 with antisense nucleotides produced greater growth inhibition in xenografts of PC3 prostate cancer cells compared to JNK1 (Yang et al., 2003), suggesting a cell type specific nature for the function of JNK isoforms. Furthermore, overexpression of constitutively active JNK resulted in cell migration and invasion of the breast cancer cell (MDA-MB-468), due to stimulation of ERK1/2 dependent c-Fos activity by the sustained JNK in cancer cells (Wang et al., 2010a). Recent studies revealed that the levels of JNK fluctuate during cell cycle progression, and JNK plays a role in G2/M phase transition via phosphorylation of cdc25C (Gutierrez et al., 2010, Gutierrez et al., 2010(a)). Preliminary studies were conducted to determine if JNK played any role in UVC mediated cell cycle regulation in MDA-MB-231 cells, but results were inconclusive. The effect of ATP could have been examined in this context. This could be due to the proliferation of MDA-MB-231 being relatively independent of serum-inducible JNK activity (Yang et al., 2003). Synchronising the cells in G1 or S phase could probably provide a clearer insight on the role of JNK and its inhibition by ATP in this cell type.

In summary, these results elucidated the inhibitory effect of ATP in TNF-α, IL-1β and UVC mediated JNK signalling in cancer cells. However the physiological effect of this inhibitory phenomenon in MDA-MB-231 cells is still unclear as ATP did not significantly alter UVC induced apoptosis. The inhibitory effect on IL-1β and TNF-α could still be important as expression of inflammatory molecules, for example COX-2 and E-selectin, which are regulated by JNK, are important in the maintenance of an inflammatory environment. Inflammation is now recognised to play a key role in cancer progression (Porta et al., 2009, Gonda et al., 2009). Nevertheless, the experiments in this chapter expanded the model established in HUVECs, suggesting that the inhibitory effect of ATP on JNK signalling is widespread.

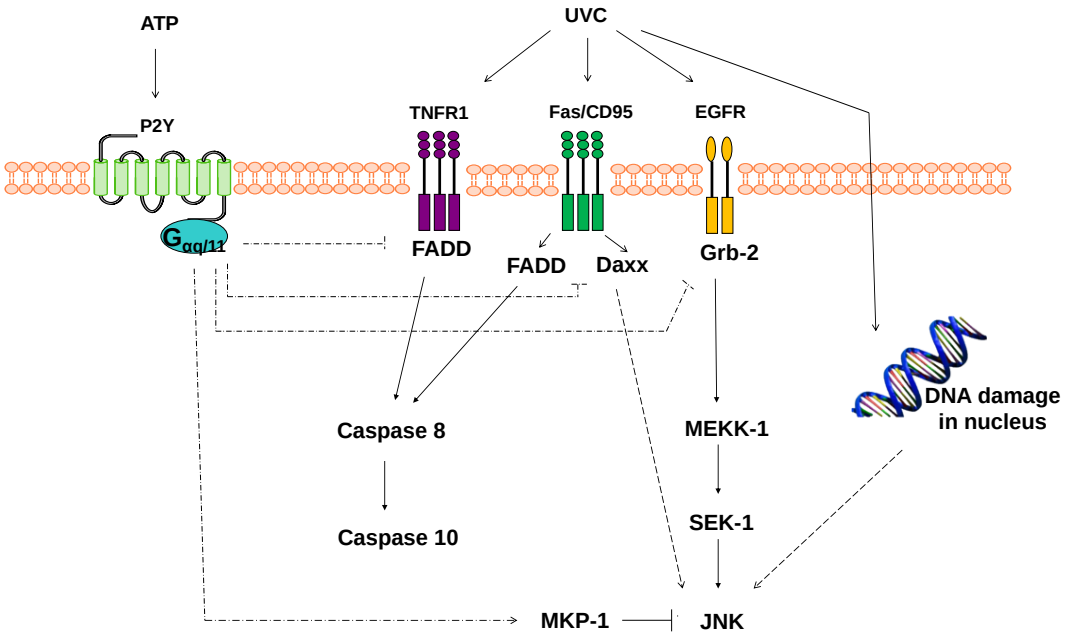

Figure 4.19: Proposed model of ATP inhibition in UVC induced JNK signalling

As discussed in section 4.7, UVC stimulated JNK activity via various mechanisms downstream of membrane receptors and DNA damage (Dunkern et al., 2001, Hamdi et al., 2005, Sheikh et al., 1998, Song and Lee, 2007, Wu et al., 2002). The probable inhibition mechanisms of P2Y receptor via $G_{q/11}$ were indicated as dash-dot lines whereas the dash lines represented that signalling intermediates might lie in between the pathway.

5.0 General Discussion

Physiologically, inflammation is a natural defence mechanism to protect the cells against microorganisms, foreign bodies and malignantly transformed cancer cells. However, this reaction is normally transient and localised. Failure of its resolution results in chronic inflammation which has been shown in the pathogenesis of various diseases, such as atherosclerosis, cancer, asthma, psoriasis and rheumatoid arthritis (Krishnamoorthy and Honn, 2006). Thus therapeutic agents that could interfere with the signalling activity induced by cytokines could be used in the treatment of inflammation-related diseases. One of the signalling pathways involved is the stress-activated protein kinase, JNK. Activation of JNK is not only linked to the pro-inflammatory effects of cytokines but it is also implicated in the pro-apoptotic effects of chemotherapeutic drugs (Cui et al., 2007, Sabapathy, 2012). In this study, endogenous GPCR ligands (ATP and adenosine) that activate purinoceptors, were found to inhibit JNK signalling induced by different pro-inflammatory mediators in various disease phenotype, such as atherosclerosis and cancer. This revealed an exciting new protective mechanism of GPCRs via $G_{q/11}$ and/or G_s downstream signalling reduced cytokine and UVC induced JNK activity.

Overall, this study strongly supports the hypothesis that activation of $G_{q/11}$–coupled GPCRs to inhibit cytokine driven JNK activity is a widespread, and possibly physiologically relevant phenomenon. Activation of purinoceptors inhibited TNF-α, IL-1β or UVC mediated JNK signalling in HUVECs, MDA-MB-231 and SW1353 cell lines. Previous studies have demonstrated that PAR_2 and H_1 receptor activation can also decrease TNF-α induced JNK signalling in different cell types (McIntosh et al., 2010, Wang et al., 2003). Thus, the protective effect of GPCRs could be a general one, rather than cell type or receptor specific. These receptors have been shown to mildly or transiently activate JNK signalling itself, especially in receptor over-expression systems (Notcovich et al., 2010, Shen and DiCorleto, 2008, Thevananther et al., 2004), therefore perhaps JNK phosphorylation is not a general feature of GPCR activation in normal cells, but is only manifest in disease phenotypes where receptor is upregulated. For example, only one breast cancer study

has clearly shown that JNK activity was required in PAR_2 induced chemokinesis (Su et al., 2009). Some primary cultured cells do produce a JNK signal but this is often only in response to high concentrations of activating agonist (Schmitz et al., 1998, Thevananther et al., 2004). Thus, GPCR induced JNK signalling in the endogenous receptor population may not produce physiologically significant effects, but its sufficient to inhibit the detrimental JNK signalling by other agents.

Based on the consistent findings that atherosclerotic lesions express higher phospho-JNK staining, in both animal models and patient samples (Chaudhury et al., 2010, Meijer et al., 2012, Wang et al., 2011), especially in the endothelial cells and cardiac myocytes of progressive and vulnerable lesions, JNK activity is termed pro-inflammatory and pro-atherosclerotic. Cardiovascular studies using primary endothelial cells (HUVECs) in chapter 3 has demonstrated that ATP and adenosine stimulated the activation of $P2Y_{11}$ and A_{2A} receptors to produce an inhibitory effect in TNF-α and IL-1β mediated JNK signalling. This effect could be mediated by both $G_{q/11}$ and G_s downstream signalling events as the inhibitory effect was partially reversed by the presence of $G_{q/11}$ inhibitor YM254890 and the PKA inhibitor, H89. However the exact inhibitory mechanism of these GPCRs is still unclear, although several probable pathways are suggested. For instance, $G_{q/11}$ could indirectly couple to PKC ζ and ε or ERK5 signalling to inhibit JNK signalling, induce the expression of MKP-1 or inhibit the recruitment of adaptor proteins to the cytokine receptors, as reported in various studies outlined in chapter 3. These studies also suggest a physiologically relevant mechanism for P2Y activation and shear stress as both produce anti-atherosclerotic effects via inhibition of JNK signalling. Yet, these studies focused on the inhibitory effects in TNF-α mediated JNK signalling, and no data has yet suggested that similar inhibitory mechanisms are exhibited for IL-1β which couples to different signal adaptor proteins. Thus, future studies using pharmacological inhibitors, siRNA and dominant negative adenovirus targeting the above signalling components could clarify their role in relation to IL-1β mediated JNK signalling.

The inhibitory effect of ATP in IL-1β induced JNK signalling is also reflected in the reduction of COX-2 expression. COX-2 mediates the production of both pro- and anti-inflammatory prostaglandins as it preferentially couples to membrane-bound PGE_2 synthase, perinuclear PGI_2 synthase and thromboxane synthase (Ueno et al., 2001). PGI_2 is a potent vasodilator with anti-platelet effects that prevents thrombus formation (Kadowitz et al., 1978, Weiss and Turitto, 1979). The protective role of PGI_2 in atherosclerosis is also indicated in an *in vivo* model of combined $ApoE^{-/-}$ and PGI receptor (IP) knockout mice (Kobayashi et al., 2004). Deletion of ApoE and IP produced larger atherosclerotic lesions in a shorter time, and higher ICAM-1 expression compared to $ApoE^{-/-}$ alone. Thus, COX-2 expression alone is not a sufficient marker for the potential anti-inflammatory effect of ATP in this study. Other JNK dependent pro-inflammatory proteins, such as E-selectin and interleukin-8, and the apoptotic proteins caspase 3 and protein phosphatase 2A could be measured, as the expression of these proteins are reduced in the presence of a JNK inhibitor (Chaudhury et al., 2010).

Nevertheless, as the inhibitory effect of ATP required the pre-activation of its cognate receptors, P2Y receptor agonists could be explored as therapeutic agents in atherosclerosis to limit the pro-inflammatory effect of cytokines. This hypothesis could be tested using *in vivo* models of combined $P2Y^{-/-}$ $ApoE^{-/-}$ mice infused with low dose TNF-α. However, endothelial cells from different regions of the vasculature express P2Y receptor at different levels. In this study, HUVECs highly expressed $P2Y_{11}$ receptor that mediated the inhibitory effect of ATP whilst the $P2Y_2$ expression was too low to mediate the ATP effect. However in human coronary artery endothelial cells, $P2Y_2$ receptor is mainly expressed, with lower $P2Y_{11}$ receptor levels (Ding et al., 2011b, Moore et al., 2001). Thus, a compound with a generalised, rather than a subtype selective P2Y agonist property could potentially be more valuable therapeutically.

The results from this study also support the usage of pathway selective agonists (Baker and Hill, 2007) that could antagonise the pro-inflammatory effect of GPCR, but still retain the inhibitory effect on cytokine mediated JNK activity. For instance, for $P2Y_1$ receptor, a double $P2Y_1^{-/-}$ and $ApoE^{-/-}$ knockout model has shown reduction in atherosclerosis compared to $ApoE^{-/-}$ alone (Hechler et al., 2008) and a decrease in P-selectin, VCAM-1 and ICAM-1 in $P2Y_1^{-/-}$ knockout compared to wild type (Zerr et al., 2011). Based on these studies that support the pro-atherosclerotic role of $P2Y_1$ receptor, a $P2Y_1$ antagonist could be developed as an atherosclerosis treatment. However, the results from this study suggest that activation of P2Y receptor for its downstream signalling mechanism is essential for the inhibitory effect of JNK activity. Hence, pathway selective agonist of $P2Y_1$ receptor could also be applied as a treatment for atherosclerosis.

The inhibitory properties of ATP and adenosine mediated JNK inhibition was also studied in breast cancer and chondrosarcoma cell lines. A concentration dependent inhibition by ATP and adenosine of both TNF-α and IL-1β mediated JNK activity was observed in MDA-MB-231 cells, with IC_{50} values lower than observed in HUVECs, whereas ATP inhibited the early phase of UVC induced JNK signalling in MDA-MB-231 and SW1353 cells. Hence these results suggest that as long as purinoceptors are expressed on the cell surface, the inhibitory effect of ATP and adenosine could be exhibited. qRT-PCR studies could then confirm the expression and their abundance of P2Y and P2X subtypes on these cancer cell lines. Adenosine, that is present in high concentrations in the tumour core (Ohta et al., 2006), could also produce similar inhibitory effects in UVC mediated JNK signalling, and this effect will be determined in future studies. Inhibition of UVC mediated JNK signalling by a JNK inhibitor or ATP did not significantly alter the apoptotic effect of UVC in MDA-MB-231 after 24 hours suggesting that the duration of study may not be long enough to reveal the changes in the apoptotic outcome. Clonogenic assay which measures the proliferative ability of cells after several days could provide a better reflection of the overall apoptotic and anti-proliferative effects of radiation, including the early apoptotic and necrotic events and also late accelerated senescence

and mitotic catastrophe (Chang et al., 1999, Erenpreisa and Cragg, 2001, Mirzayans et al., 2005, Roninson et al., 2001). The effect of irradiation with UVC upon cancer cells fixed in S phase, with or without the presence of a JNK inhibitor or ATP, could also be performed to examine if JNK inhibition affects cell cycle progression after UVC treatment.

Furthermore, γ-radiation which is widely used in combination with surgery and chemotherapy to treat cancer (Nias, 1976, Schulz-Ertner et al., 2006, Veldeman et al., 2008), has also been shown to activate JNK signalling to induce apoptosis in various cell lines, including Jurkat T cells, blood lymphocytes, epidermoid carcinoma A431, myeloid leukimia U937 and MDA-MB-231 (Chen et al., 1996a, Dent et al., 1999, Kharbanda et al., 1995, Reardon et al., 1999). This JNK activity is dependent on EGFR, TNF-R1 and TGF-α signalling (Dent et al., 1999, Reardon et al., 1999). Based on the similarities in the signalling mechanisms of γ-radiation and UVC induced JNK signalling, it is hypothesised that P2Y activation could also inhibit γ-radiation mediated JNK activity. Moreover, application of the ERK inhibitor, PD98059 augmented the γ-radiation induced JNK signalling within 30 minutes (Reardon et al., 1999), suggesting that ATP activation of ERK signalling could produce a reduction in JNK activity. In addition to apoptosis, γ-radiation also induces G_2/M cell cycle arrest in breast cancer cells (MCF-7) and this was reduced via pre-treatment with the JNK inhibitor, SP600125 (Mingo-Sion et al., 2004).

Recent studies also revealed another phenomenon mediated by radiation therapy, named the bystander effect, whereby irradiated cells induce DNA double-strand breaks in neighbouring non-irradiated cells (Baskar, 2010, Prise et al., 2003, Sokolov et al., 2005), leading to peripheral cell damage such as inflammation of endothelial cells that results in higher risk of cardiovascular-related diseases (Little et al., 2010, Boerma and Hauer-Jensen, 2010). It has been suggested that these DNA damaging signals are transmitted via gap junctions and/or release of soluble factors (Baskar, 2010, Prise et al., 2003). Transforming growth factor-α is released by irradiated cells

to increase JNK signalling and reactive oxygen species in the neighbouring cells to induce apoptosis (Dent et al., 1999). ATP is also implicated as γ-radiation induces release of ATP to stimulate intracellular Ca^{2+} increase and ERK signalling in neighbouring cells (Tsukimoto et al., 2010). Incubation of apyrase that degrades extracellular ATP reverses the bystander apoptotic effect in UVW glioma cells (personal communication with Dr Marie Boyd). Based on the results from this study, it is hypothesised that application of a P2Y agonist is desired to systemically protect the cells from the detrimental effect of γ radiation, and targeted delivery of a P2Y antagonist to the tumour site could enhance the apoptotic effect of radiation therapy. Nevertheless, experiments using a P2Y antagonist combined with radiation treatment for *in vitro* cancer cell lines and *in vivo* mice cancer models needs to be undertaken to support this idea.

In addition to radiotherapy, the localised high concentration of ATP and adenosine in the cancer cell environment could also activate P2Y receptor to interfere with chemotherapeutic efficacy. Chemotherapeutic drugs of different pharmacological actions are known to induce persistent JNK signalling for their apoptotic effect. This includes DNA damaging agents (cisplatin, mitomycin C, cytarabine, etoposide and adriamycin) and microtubule-interfering agents (MIA) (paclitaxel, docetaxel, vinblastine and vincristine) (Osborn and Chambers, 1996, Saleem et al., 1995, Sanchez-Perez et al., 1998, Wang et al., 1998b). JNK activity is required for their apoptotic action as persistent JNK signalling is neither observed in drug-resistant cell lines nor by the inactive structural derivatives of drugs (Sanchez-Perez et al., 1998, Wang et al., 1998b). The exact JNK signalling pathway for these drugs are unclear, but MIA is reported to stimulate Ras and ASK1 to induce JNK activity (Wang et al., 1998b). Thus, further experiments could study the combined effect of ATP and adenosine with these chemotherapy drugs.

In conclusion, results from this study revealed great potential in manipulating the activation of P2Y in order to regulate the overall pro-inflammatory JNK signalling in

inflammatory-related diseases such as atherosclerosis and cancer. Activation of P2Y could induce mild and transient JNK activity, which then reduced the detrimental high magnitude and prolonged JNK signalling mediated by cytokines and UVC. Using a full P2Y agonist would allow maximal activation of both pro- and anti-inflammatory pathways, in contrast a pathway selective agonist of P2Y receptor could minimise the pro-inflammatory signalling, but still maintain the anti-inflammatory property of inhibiting cytokine and UVC induced JNK signalling. In addition, a P2Y antagonist could be used in conjunction with radiation and possibly chemotherapy to prevent the endogenous ATP in the tumour cells inhibiting the therapeutic benefit of the JNK signalling. Thus, the targeted delivery of these P2Y pathway selective agonists and antagonists to the therapeutic site is highly desirable. Further *in vitro* and *in vivo* studies are required to validate these new conceptual therapies.

6.0 References

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